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TITLE: Research and Operational Support for the Study of Militarily Relevant Infectious Diseases of Interest to the United States and Royal Thai Government

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PI - Signature Date
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Bangkok 10400, Thailand

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I. INTRODUCTION

A. General

Collaborative studies into infectious diseases of military importance have been conducted at the Armed Forces Research Institute of Medical Sciences (AFRIMS) by both the US Army Medical Component (USAMC) and the Royal Thai Army Medical Component (RTAMC) for 4 decades. Studies leading to develop drugs and vaccines to combat tropical diseases of military relevant importance.

B. Statement of work

Administrative, logistical and scientific personnel required to support the ongoing US Army AFRIMS research efforts, and utilities and maintenance required to support the US Army AFRIMS research effort.

C. US ARMY AFRIMS research efforts at Department of Entomology

Department of Entomology research efforts are the following:

1. Use of GIS to Assess Relative Risk in Different Biotopes within Endemic Scrub Typhus Areas
2. Use of a Geographic Information System (GIS) to Implement and Evaluate the Efficacy of Targeted Vector Control as a Means of Reducing Malaria Ttransmission
3. Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines
4. Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

D. US ARMY AFRIMS research efforts at Department of Immunology

Department of Immunology research efforts are the following:

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E. US ARMY AFRIMS research efforts at Department of Enteric Diseases

Department of Enteric Disearses research efforts are the following:

1. Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand
2. Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens
3. Travelers' Diarrhea among US Forces Deployed for Operation Cobra Gold
4. Characterization of Enteric Pathogens Isolated from Children in Phnom Penh

F. US ARMY AFRIMS research efforts at Department of Veterinary Medicine

Department of Veterinary Medicine research efforts are the following:

1. Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta*)/*Plasmodium cynomolgi* Malaria Models
2. Care and Maintenance of Rhesus (*Macaca mulatta*) and *Cynomolgus (Macaca fascicularis)* monkeys and Management of Breeding Colonies
3. Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/Quality Surveillance Program
4. A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs
5. Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelli*) for Screening Exoerythrocytic Antimalarial Drugs

G. US ARMY AFRIMS research efforts at Department of Virology

Department of Virology research efforts are following:

1. The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue
2. A Recombinant Hepatitis E Vaccine Efficacy Study In Nepalese Volunteers
3. Prospective Study of Dengue Virus Transmission and Disease in Primary School Children
4. A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Children
5. A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Infants
7. Influenza Surveillance in Southeast Asia

H. US ARMY AFRIMS research efforts at Department of Retrovirology

Department of Retrovirology research efforts are following:

1. Screening and Evaluation of Potential Volunteers for a Preventive HIV-1 Vaccine Trials in Thailand (RV148, HSRRB Log No.)
2. A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp 120 B/E (AIDSVAX® B/E) Boosting in HIV-uinfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795)

I. Space and Utilities Required

Funding under the cooperative agreement is also directed by the Principal Investigator to the provision of site maintenance including space and utilities management for both the RTAMC and the USAMC in support of research activities.

II. BODY

A. Department of Entomology, AFRIMS FY04 Research Accomplishments

1. Title of research project: Use of GIS to Assess Relative Risk in Different Biotopes within Endemic Scrub Typhus Areas

a. Investigators:

LTC James W. Jones, PhD;
Kriangkrai Lerdthusenee, PhD
Joe Nigro, MS

b. Objectives:

Geographic information systems (GIS) will be used to explore the spatial relationships of rodent, chigger, and Orientia populations and to assess risk in individual habitats.

c. Methods:

GIS has been used extensively to analyze remotely- sensed data and to predict distributions of plant and animal species. In our study, rodent-hosts and chigger vectors will be collected from residential, rice field, forest edge, and forest habitats in areas highly endemic for scrub typhus. Vegetation distribution within each habitat will be characterized. In each habitat we will establish 2 trapping systems: a transect line of 100 meters and a grid system of 10x10 meters, each consisting of 20 traps. Captured rodent specimens will be identified to species and blood and tissue samples collected. Ectoparasites will be removed from trapped rodents and chigger specimens will be collected for identification and for Orientia assay. Data will be entered into GIS which will be used to define spatial relationships between rodent, vegetation, chigger and Orientia species. The final product will be a predictive tool for scrub typhus risk in biotopes within an endemic scrub typhus area.

d. Results (accomplishments during the period of May 2004 - December 2004):

High resolution satellite imagery has been acquired, processed, and incorporated into ArcGIS in order to analyze rodent and chigger distributions and to identify environmental variables that contribute to suitable ecological conditions for these species. These environmental variables are, in turn, indicative of the presence of scrub typhus. The Quickbird images of the three major study sites (Mae Sad, Mae Chan, and Bangkruai) are allowing for the extraction of geographic, topographic, and environmental information pertaining to these areas. The environmental information being collected through the imagery, as well as, field campaigns consists of vegetation type, density, soil moisture, elevation, and slope. Eventually, this data will be correlated with the data from rodent and chigger collections and passive case detection of

scrub typhus. In this study, rodent-hosts and chigger vectors are being collected from different biotopes associated with endemic scrub typhus areas throughout Thailand. These collection locations include residential areas, rice fields, forest edges, and forest habitats where scrub typhus is endemic. Scrub typhus occurrence is marked by the presence of these fringe habitats where forested areas, rice fields, and villages meet to form an ecotone. Remote sensing and GIS provide the optimum means for identifying these areas and quantifying them. Using higher resolution satellite imagery (ranging from 0.6 meters to 2.5 meters depending on the bands being used) more detailed information will be extracted within each habitat ultimately producing a more statistically accurate risk model. In each habitat, we established 2 trapping systems: a transect line of 100 meters around the house and a grid system of 10x10 meter, each consists of 20 traps (10 small live-traps, 10 medium live-traps). Captured rodent specimens are being identified to species. Blood and tissue samples are collected from animals, kept in cryovials, and stored at -70° C. Each blood and chigger sample is assessed by ELISA and positive samples are assessed by polymerase chain reaction (PCR), with primers specific for Orientia. Samples that are identified as positive for Orientia are examined for further strain identification. All ectoparasites are removed from trapped rodents for identification. Chigger specimens were collected and identified, along with the plant species found in the trap areas. Plant and rodent diversity is governed not only by physical factors found at a site, such as climate, soil type, and elevation but also by local land usage. Classification of local vegetation is being undertaken using state-of-the-art eCognition classification software and will permit correlation of vegetation types with predicted distribution of individual rodent species. We also anticipate that this will allow us to associate vegetation types with animal species regionally. Vegetation density will also be calculated using the normalized difference vegetation index (NDVI).

e Future plans:

Pending funding in FY05.

2. Title of research project: Use of a Geographic Information System (GIS) to Implement and Evaluate the Efficacy of Targeted Vector Control as a Means of Reducing Malaria Transmission.

a. Investigators:

LTC James W. Jones, Ph.D
Dr. Gabriela Zollner, Ph.D
Dr. Jetsumon (Sattabongkot) Prachumsri, Ph.D
Dr. Ratana Sithiprasasna, Ph.D
Dr. Benjawan Khuntirat, Ph.D
LTC Robert Scott Miller, MD

b. Objectives:

This project was a continuation of the Ban Kong Mong Tha study in 2003. The focus is on evaluation of the efficacy of vector control techniques to interrupt malaria transmission. Established control strategies that were evaluated include use of permethrin-treated bednets and use of residual deltamethrin spray. Novel strategies that were evaluated include use of Mosquito Magnet trap to eradicate mosquitoes within a specific area, with impact on vector populations and malaria prevalence determined. We will continue to use GIS to evaluate mosquito (adult and larval), human, and parasite populations. This site will serve as a core site for development of a malaria vector control system.

c. Methods:

1. Site Mapping: LANDSAT images (30 meter resolution) of the village and surrounding area have been acquired and used to establish the GIS using ERDAS software. IKONOS images (1 meter resolution) of the study site are on order.

2. Adult Mosquito Collections: Each month, adult Anopheles mosquitoes are collected while attempting to bite collectors at selected sites throughout the village. The village has been divided into 11 grids (A-I, School, and Wat) -- monthly collections are made at 8 separate sites (in each in Grids A-H). Mosquitoes are identified to species, parity determined, and the abdomen and thorax of each individual mosquito tested by ELISA for P. falciparum, P. vivax-210, and P. vivax-247 circumsporozoite protein. The geographic and temporal distribution of each species is mapped out. A total of 9,618 adult anophelines representing 29 species have been collected during landing/biting collections. A total of 8,457 mosquitoes have been tested by ELISA, with 17 positive mosquitoes (0.2% infection rate). Mosquitoes were infected with P. falciparum, P. vivax 210, P. vivax 247, and mixed P. vivax 210/247.

3. Larval Mosquito Collection: Each month, larval Anopheles mosquitoes are collected within the village and surrounding areas to determine if there is an association between larval mosquito habitat and adult mosquito distribution in the town. GPS is used to mark the site of each larval mosquito collection. Larvae are returned to AFRIMS and emerging adults are identified to species. To date (Jun 99-Sep 01), a total of 7,203 larval Anopheles mosquitoes representing 32 species have been collected, reared to adults, and identified.

4. Human Malaria Surveillance: A human use protocol for this project received final approval from the US Army HSRRB and the Thai Ministry of Public Health in April, 2000. Villagers were entered into the study beginning in May, 2000, when baseline demographics and whole blood (200ul/person) from the entire volunteer population was obtained by fingerprick. During each subsequent month (currently May 2000-Sept 2001), 50-75% of the cohort was evaluated for malaria by fingerprick (thick- and thin-blood smears) and dipstick assay to determine Plasmodium prevalence. Additional blood samples were stored on filter paper and in microcapillary tubes for subsequent genetic analysis of parasites and determination of transmission-blocking antibody levels. Individuals positive for malaria in any given month have a 2.5-ml (individuals <10 years old) or 5-ml (individuals > 10 years old) blood sample drawn by venipuncture within 24 hrs of the finger-prick. Approximately 1-2ml of this blood sample is fed to mosquitoes in a membrane feeding system (this allows us to determine which particular individuals are capable of infecting mosquitoes at that point in time). Duplicate thick/thin blood

smears are made at the time of the mosquito feed. An additional sample (1-2 drops) is stored on filter paper for PCR analysis for malaria. The remainder of each blood sample is separated into 0.1ml aliquots for evaluation of factors that might affect transmission. The goal is to determine the focality of transmission by tracking the spread of specific Plasmodium clones through the human population.

5. Establishment of the GIS and malaria modeling: Mapping out of the village (houses, road, building, rivers) and larval mosquito habitats has been completed. Current effort focuses on incorporating data (adult and larval mosquito collections, # of people per house, blood films collected, positive blood films, vegetation, etc.) into the GIS. Dr. Don Roberts and Mrs. Penny Masuoke in the Department of Preventive Medicine, USUHS, are collaborators on this project and are currently establishing the GIS. In addition, Dr. Richard Kiang at NASA is developing a plan for modeling of the interaction of vector mosquitoes and actual malaria.

6. Implementation of Malaria Control: Current effort has focused on evaluating the impact of the Thai Ministry of Public Health effort to spray all houses in the village with 5% Deltamethrin. Houses were sprayed in March and October 2000 and March and July 2001. In addition, permethrin-treated bednets were provided to 123 families in the village. Analysis suggests that neither of these two control methods had a significant impact on either vector populations or malaria rates.

In FY04: Collection of human/parasite/mosquito data continued as described above. Key components of the GIS include remote sensing data (vegetation and water sources), environmental (daily temperature, relative humidity, rainfall), demographic (house information, age, sex), parasitological (parasite species distribution, gametocyte rates, etc.), and entomological (adult and larval distribution and habitats as well as entomological inoculation rates) indices. Cluster analysis was used to identify relationships between malaria cases and adult mosquito distribution, and between adult mosquito distribution and larval distribution. The GIS was then used to identify key sites for intervention (larval and adult mosquito control, as well as treatment of malaria reservoirs with transmission-blocking antimalarials). The different grids in the town are randomly selected as control or treatment sites. Control sites receive no treatment, other than that normally provided by the Ministry of Public Health (normally chloroquine or mefloquine treatment of clinically-ill P. vivax or P. falciparum patients, respectively. The impact on malaria transmission within the village was determined. Subsequent to this study, we evaluated the efficacy of using the American Biohysics Corporation Mosquito MagnetTM (also known as the Counterflow 2000) Pro Model trap to control vector populations in selected areas within the village. Use of this trap has been advocated as a means of eliminating mosquitoes within an approximately 1-acre area.

d. Results (accomplishments during the period of January 2004 - December 2004):

Project completed as scheduled. Collection of human and parasite data continued through July 2004 as described for the Ban Kong Mong Tha study. The design of the Geographic Information System (GIS) was completed in Apr 03. Key components of the GIS include remote sensing data (vegetation and water sources), environmental (daily temperature, relative humidity, rainfall), demographic (house information, age, sex), parasitological (parasite species

distribution, gametocyte rates, etc.), and entomological (adult and larval distribution and habitats as well as entomological inoculation rates) indices. Cluster analysis (conducted by the USUHS team) has shown that there is no evidence of association between the location of malaria cases and distance to adult mosquito collection sites (ANOVA), distance to larval breeding sites (ANOVA), or land use patterns, including water bodies (Wilcoxons Rank Sum test). For associations of malaria incidence with environmental factors, 30, 60, or 90m buffer zones were drawn around groups of houses that contained at least one malaria-positive individual. Further analyses to identify associations of P. falciparum or P. vivax cases and location of Plasmodium-infected/infective mosquitoes are ongoing. Preliminary results suggest that there is no association due to the low numbers of Plasmodium-positive anophelines caught (approx. 0.3%). Currently the GIS is being used to create a model of malaria transmission dynamics in the study area which can be used to predict future malaria incidence using a minimal amount of epidemiological data. Primary efforts also focused on the evaluation of the efficacy of the American Biophysics Corporation Mosquito Magnet Pro Model trap to control vector populations in selected areas within the village. This portion of the study was initiated in May 02 and continued through Dec 04. The schedule for the study was as follows:

May-Jun 2002: Pre-treatment months. Surveillance conducted without any traps.
Jul-Dec 2002: Treatment months (Phase I). 2 control grids (B/H) and 2 treated grids (D/F).
Jan-Jun 2003: Control months (no traps set out)
Jul-Dec 2003: Treatment months (Phase II). 2 control grids (D/F) and 2 treated grids (B/H).
Jan-Jul 2004: Follow-up months to evaluate residual impact on vector populations.
Aug-Dec 2004: Data analysis

Use of the Mosquito Magnet (MM) trap has been advocated as a means of eliminating mosquitoes within an approximately 1-acre area. The military expressed an interest in using the MM trap for vector control. Grids B, D, F and H of the village are used as sites for this evaluation. In the first year of the study, Grids D and F were selected as treatment sites, and Grids B and H served as control sites. In the second year of the study, Grids D and F served as control sites and Grids B and H were treated sites. Three MM traps were set up in each of the two treatment areas (4 traps in Grid H), which were located within 50 meters of each other. Traps were run continuously (using propane and octenol packets as recommended by American Biophysics Corporation) for 6 months. Landing/biting (L/B) collections (2 people/house) were conducted monthly in a total of 6 houses in each treatment and control grid (6 houses per grid x 4 grids = 24 houses total). For 2 nights each month, mosquitoes were collected from 3 of the 6 houses in each grid on nights 1 and 3, and from the remaining 3 houses on nights 2 and 4. This means that was a total of 12 collections made per grid every month. Collections were made from 1800 to 2400 each night (our data indicate that approx. 70% of all mosquitoes are collected before 2400 hours). Collections using these procedures commenced in May 2002, with the MM traps switched on in Jul 02 and switched off in Jan 03 (Phase I). We switched the traps on again in Jul 03 (control and treated areas reversed), and traps were switched off in Jan 04 (Phase II).

L/B collections in the four grids comprised 10,001 anopheline mosquitoes, of which approx. 0.3% (including 16 An. maculatus, 8 An. sawadwongporni, and 7 An. minimus mosquitoes, or pools of up to five mosquitoes) were positive for Pf or Pv. During Phase I (Jul-Dec 02), MM trap catches comprised 560 anopheline mosquitoes, including 275 (49%) An. kochi and 184 (33%) An. maculatus mosquitoes. During Phase II (Jul-Dec 03), MM trap catches comprised 304 anophelines, including 128 (42%) An. barbirostris, 50 (16%) An. kochi, and 46 (15%) An. minimus mosquitoes. The difference in magnitude between catches for L/B collections compared to MM trap catches (operated during the same period) suggests that the traps were not effective in trapping adequate numbers of mosquitoes. However, the species composition was comparable to that of L/B collections, and hence the MM trap remains a useful surveillance tool for anopheline vectors of malaria.

Malaria prevalence and MM trap catches differed according to vector abundance and season, respectively. The prevalence (%) of human Pf cases was slightly higher in control grids compared to treated grids in each of the paired study areas during Phases I and II, but this finding was not significant. During Phase I of treatment (Jul-Dec 02), overall P. falciparum prevalence was 25% lower in treated areas (3.1%) vs. control areas (4.3%). During Phase II of treatment (Jul-Dec 03), P. falciparum prevalence did not differ between treated and control areas (2.6% and 2.8%, respectively).

Analysis of the data set from the Ban Kong Mong Tha study is almost complete. AFRIMS collaborated with the Thai Ministry of Public Health to further evaluate the efficacy of residual house spray and the impact of the treated bed-nets on the mosquito populations (Jun 1999-Jan 2002). Neither residual house spraying nor use of permethrin treated bed nets had a marked impact on mosquito populations or malaria prevalence rates. One reason for this is that the anopheline malaria vectors in the Ban Kong Mong Tha study area are generally exophagic and exophilic. It is possible that individuals may become infected during the 2-3 hours before bedtime, i.e. during the time that people move between houses, watch TV, etc. These results raise important questions regarding malaria vector control strategies in Thailand and other countries in SE Asia where the same anopheline vector species are present.

e. Future plans:

Completed.

3. Title of research project: Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines

a. Investigators:

LTC James W. Jones, Ph.D
Dr. Kriangkrai Lerdthusenee, Ph.D
Dr. Benjawan Khuntirat, Ph.D

b. Objectives:

1. Conduct genetic characterization of O. tsutsugamushi infecting 12 colonies of Leptotrombidium chiggers sps. maintained at AFRIMS.

2. Evaluate the ability of each of the 12 chigger colonies to transmit O. tsutsugamushi to laboratory mice. Down-select 4-5 key chigger colonies for further studies. These chigger colonies should be infected with different strains of O. tsutsugamushi and should produce consistent, high infection rates when fed on mice.

3. Focus efforts on building up down-selected chigger colonies to the high levels required for potential vaccine studies.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Criteria used to assess efficacy must include quantification of rickettsemia in the mice; however, additional methods (clinical or immunological responses) may also be assessed.

c. Methods:

1. Characterization of Strains/Isolates of Orientia tsutsugamushi: The goal is to characterize the 12 strains of Orientia tsutsugamushi infecting our 12 chigger colonies in order to determine phenotypic and genotypic relationship between different strains

2. Evaluate the efficacy of chigger colonies to transmit O. tsutsugamushi to mice and down-select 4-5 key colonies: Colonies of mites were characterized by PFGE and DNA amplified fingerprinting to identify colony lines with the highest rate of infectivity to laboratory mice. These colonies are being monitored over several generations to ensure a "hot" strain capable of infecting >90% of recipient animals is maintained. In addition, control lines of uninfected chiggers have been monitored. This ensures that the mite colonies that are the best vectors of scrub typhus to be used in the mouse protection model. Additional studies need to be conducted to determine if intrathoracic inoculation of O. tsutsugamushi into uninfected chiggers can be used as a means of developing new colonies of infected chiggers. The ability to rapidly develop colonies of chiggers infected with newly identified strains (to include antibiotic resistant strains) of O. tsutsugamushi allows us to evaluate vaccine candidates against a wide variety of pathogens. Finally, we attempt to characterize mechanisms by which Leptotrombidium deliense and L. chiangraiensis become infected with O. tsutsugamushi, to include analysis of vertical (mite to mite) and horizontal (vertebrate to mite to vertebrate) transmission. The ability to infect mites from infected hosts would allow us to evaluate the ability of potential vaccine candidates to prevent the transmission of O. tsutsugamushi from mites to vertebrates and subsequently back into mites. This ability of a vaccine candidate could have important epidemiological implications.

3. Build-up key chigger colonies to levels sufficient to support vaccine challenge studies: The rearing and maintenance of Leptotrombidium chiggers is a long, slow process. The total life cycle (from egg to egg-laying adult) requires approximately 3 months (this is in contrast to 2-3 weeks for most mosquitoes). Each female chigger will only produce about 1000 eggs over her lifetime. Once a chigger colony is selected for use in vaccine trials, it requires approximately 6 months to build it up to a level required to support the trial.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Initial efforts focus on determining the course of rickettsemia over time for the 4-5 strains of O. tsutsugamushi selected for further study and on the development and/or confirmation of diagnostic procedures (PCR, ELISA, etc.) to quantify rickettsemia in challenged mice. We will also evaluate the effect of chigger infection with specific strains of O. tsutsugamushi on potential indicators of immunity, to include lymphocyte transformation, morbidity (as quantified by food consumption, weight gain/loss, activity, etc.), and mortality (time to death following chigger infection).

d. Results:

The chigger-Challenge model's SOP is completed. The Animal-use protocol for the chigger-challenge mode is compleded.. Continuing to sequence the 56kDa polypeptides of the Lc-5 isolate from Leptotrombidium chaingraiensis and the other 3 O.tsutsugamushi isolates of Li-3, Li-4 & Li-7 (of the Leptotrombidium imphalum colonies). Establishing the procedures for quantifying the rickettsemia by the Real-time (RTQ) PCR of the Orientia tsutsugamushi Lc-1 strain, the isolated strain from the Leptotrombidium chiangraiensis. This particular O.tsutsugamushi isolate was a Karp-like strain. The completion of the new Biosafety Level-3 laboratory was delayed and as a result this evaluation. A bio-safety certification for the Biosafety Level-3 Room was conducted and passed in December 2004. Mouse-challenges will begin soon. The renovation of the new Ectoparasite laboratory in the Veterinary Medicine Building has been completed. We are, preparing to vaccinate mice according to the procedures established by the NMRC team..

e. **Future Plans:**

Conduct mouse-challenge evaluation of candidate vaccines in FY05.

4. Title of research project: Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

a. Investigators:

LTC James W. Jones, PhD.
Dr. Jetsumon Prachumsri, Ph.D

b. Objectives:

Establish procedures to produce qualified and reliable sources of Plasmodium vivax parasites to support a human challenge model.

c. Methods:

1. Validation of basic system to provide sporozoite-infected mosquitoes in support of STEP/STO requirements. This system will be based on our current ability to feed mosquitoes directly on P. vivax-infected patients reporting to local malaria clinics. In brief, adult patients report to local malaria districts when they think they may have malaria. Ministry of Public Health (MOPH) personnel make thick blood smears and check the patients for malaria. As part of an approved Human Use Protocol, Department of Entomology personnel allow to draw 20 ml of patient blood to feed mosquitoes using membrane feeding technique. These studies are conducted weekly at Mae Kasa and Mae Sod malaria clinics. Aliquote of each blood sample will be spotted on filter paper and smeared on glass slide. Confirmation of parasite species will be accomplished by PCR of filtered blood and microscopic examination of blood smears. Infected mosquitoes are returned to AFRIMS and maintained in the AFRIMS insectary. Five to 10% of mosquitoes from each mosquito feed are checked for the presence or absence of oocysts approximately 7-10 days after infection. These mosquitoes are thereafter available for use in malaria sporozoite challenge studies.

2. Refined Sporozoite Challenge System (this study was postponed from 2002 to 2003 due to delay in approval of protocol for human challenge study). In 2004 we will continue refining the system in order to reduce the variability in the mosquito infections (critical for ensuring consistent challenges) and to eliminate the risk of concomitant mosquito infections. The goal will be to develop a system that will i) consistently provide mosquito infection rates with >60% of blood-fed mosquitoes having +3/4 (>100 sporozoites) salivary gland infections, and ii) provide P. vivax-infected mosquitoes that do not harbor concomitant pathogens. Consistency in the challenge is a critical component of any vaccine trial. Plasmodium vivax-infected patients reporting to local malaria clinics will serve as the starting point for development of the "refined system". Mosquitoes will be fed on venous blood provided to them in an artificial membrane feeding system. A series of carefully controlled experiments will be conducted using the membrane feeding system. We hypothesize that pooling blood from infected patients will reduce inherent variability in mosquito infections. In addition, pooling blood from several patients may offer the added benefit by increasing the genetic diversity of the sporozoite challenge, and thus may more truly evaluate the efficacy of any candidate vaccine. Replacement of Patient Sera with Commercial Sera: Blood will be collected from patients, and packed red blood cells separated from the sera and subsequently reconstituted with commercial sera. The reconstituted blood will be fed to mosquitoes in a membrane feeding system and mosquito infections quantified. This method has the advantage of removing anti-malaria antibody that may affect gametocyte infectivity (2) and replaces patient sera that is potentially infected with concomitant acellular pathogens with commercial sera that is known pathogen-free.

3. Parasite Characterization: In the absence of an in vitro culture system, it will be necessary to feed mosquitoes on a P. vivax-infected volunteer or the blood from a volunteer. Since it will be impossible to ensure that mosquitoes are infected with a single P. vivax clone (as is currently done with P. falciparum), it is critical that we develop a method of characterizing the parasites (i.e., genetic diversity of the parasites, resistance to antimalarial drugs, etc.). Once mosquitoes are infected, parasites from the infectious blood meal will be characterized by PCR using polymorphic gene targets, such as the nonapeptide repeat region of the circumsporozoite protein (PvCSP), and the region between interspecies conserved blocks 5 and 6 of the merozoite surface protein (PvMSP1)(3,4,5).

4. We will continue our effort on establishing of continuous culture of P. vivax and verifying our technique for short term culture to provide infective gametocyte for mosquito feeding. Use of short term culture technique to produce gametocyte and study gametocyte infectivity. In this portion of the study we propose to evaluate the ability of produce infective gametocyte by in vitro culture of cryopreserved blood to infect mosquitoes. Effort will focus on establishment of cryopreservation techniques that will maximize parasite viability. Development of procedures to culture infective gametocytes would allow for production of sporozoites from P. vivax specimens obtained from throughout the world. These sporozoites could then be used in a variety of experimental models (i.e., we could use sporozoites obtained from East Timor in our hepatoma cell model to evaluate resistance of Exo-erythrocytic stage parasites to primaquine and tafenoquine). We have used reticulocyte enriched from cord blood as a host for the parasites in our system. One third of P. vivax culture maintained in McCoy'5A medium with 25% AB serum and human reticulocytes can survived up to 37 days in our system. Induction of gametocyto-genesis using McCoy'5A medium with 25% AB serum containing hypoxanthine for the culture of P. vivax will done on at least 100 cases of blood in 2004. The gametocytes from culture will be fed to mosquitoes to verify their infectivity and compare with gametocyte infectivity of the same isolates freshly fed to the mosquitoes without being cryopreserved and cultured.

d. Results (accomplishments during the period of January 2004 - December 2004):

We studied the number of erythropoietic cells determined as colony forming units from the stem cell culture initiated by co-cultivation of cord blood stem cells with mesenchymal stem cells, MSC. At present, we can keep the co-cultivated cells growing 4-6 weeks. The cord blood cells grow consistently on the MSC. We are trying to enumerate and identify the cells from this culture to confirm whether they are hematopoietic cell progenitors. The method used is phenotyping of the cells by flow cytometry. We used monoclonal antibodies to CD34 and CD133 to determine surface markers of the cells retrieved from the MSC-cocultures to verify status of the hematopoietic progenitor. Cells which bare CD133+ and CD34+ will indicate the early stage hematopoietic progenitors which can be propagated in a long-term culture. We are having some problems retrieving cells from these culture wells in order to perform flow cytometry.

e. Future plans:

Continue in FY05. The adherence between these cells and the MSC feeder layer appeared to be stronger than we expected. One alternative is to use the CD133 cell isolation kit [Mac] to separate the starting cells before cocultivation. Propagation of the cord blood stem cells in our study required contact with the MSC when compared with the culture using spent supernatant of the MSC. However, we will also try another alternative of Transwell culture plate to make good separation between these cells.
expanded to another targeted site in SE Asia. Lastly, we anticipate an expand role in regional malaria surveillance with a combination of in vivo, in vitro and genetic methods to define expanding malaria drug resistance.

B. Department of Immunology AFRIMS FY04 Research Accomplishments

1. Title of research project:

Number	Projects	Status
1	MRDD Phase III (687-2001)	In life completed 2002; report writing in progress
2	MRDD Phase IIIb – Venous vs. Fingerstick	In life completed 2003; data analysis in progress
3	Comparison of PCR adjusted HRP immunoassay vs conventional microscopy for Pf diagnosis	Completed; manuscript prepared
4	MRDD Comparison to LC-MS and PCR analysis	Protocol approved
5	Development of new cost effective HRP2 ELISA for Pf malaria drug sensitivity testing	Manuscript submitted
6	Human Malaria Vivax Challenge	Protocol in development
7	RTS,S/TRAP Vaccine in Rhesus Neonates	completed; paper published
8	Rhesus PfMSP-1 Vaccine –Safety/Immunogenicity	completed; paper published
9	Rhesus Pf AMA-1 Combinations Vaccine	Phase I completed; amendment in progress
10	Immunologic studies of SIV/p27 adenovirus as a model system for future malaria antigen studies	Protocol written; awaiting materials transfer from commercial partner
11	Quinone reductase as target for antihypnozoite activity of chloroquine and primaquine	ILIR funded; data collected
12	PvDBP Polymorphisms	PhD project in progress

13	Dengue activates plasmacytoid DCs	Completed and published
14	Schizont activates plasmacytoid DCs	Completed; manuscript submitted
15	Tafenoquine Radical Cure Dose Ranging Part II	Paper published
16	Tafenoquine Measurement – in Prophylaxis	Completed; published
17	Tafenoquine Cure/Radical Cure Dose Ranging NIH	In life completed Jan 2005; manuscript preparation in progress
18	Phase II Assessment of Azithro-Quinine Combos	In life completed; results reported at ASTMH
19	Azithro-Quinine and Azithro Artesunate in Pf Rx	Trial in progress; in life completion estimated early 2005
20	AS Bioequivalence study (comparing WRAIR IND formulation vs. Guilin non GPO product)	Protocol prepared; scientific approval obtained
21	Bioassay/HPLC/LC-MS Validation - FDA	AS complete; LC-MS In progress
22	Artesunate Phase I Protocol Development	In progress; in life anticipated early 2005
23	IV Artesunate/Methylene Blue Efficacy in Rhesus P. cynomolgi model	In life completed; data analyzed, manuscript in progress
24	Fever Surveillance in Thong Pha Phum	Protocol preparation in progress; partners contacted
25	Fever Surveillance in Sangklaburi	Results reported to local health authorities; manuscripts in preparation
26	Cambodia Malaria Prevalence Study	In life and slide reading completed; data analysis in progress
27	Leptospirosis in Sangkhlaburi	Manuscript published; second in progress
28	Field validation of New In Vitro Sensitivity Assay	Completed; manuscipt submitted
29	Molecular Assessment of Nepal Malaria Isolates	In life and data analysis completed; manuscript prep in progress
30	Bangladesh In Vitro Pf Resistant assessment	Protocol approved; First of 5 year project complted
31	Cambodia In Vitro Pf Resistant assessment	Planning initiated

32	Thailand In Vitro Pf Resistant assessment	ongoing
33	PF MSP-1 Genotyping – assay development	in progress;
34	Vivax Genotyping - PV Mahidol	Data analysis
35	Gametocyte Production for Entomology	In Progress

a. Investigators:

Dr. Mark Fukuda, MD
Dr. Fernando Guerena, MD
Dr. Robert S. Miller, MD
Dr. Victor Melendez Ph.D.
Dr. Sathit Pichyangkul, Ph.D.
Dr. Paktiya Teja-Isavadharm Ph.D.
Dr. Krisada Jongsakul, MD
Dr. Harald Noedl MD, PhD
Dr. Ruth Ellis MD
Dr. Delia Bethell, MD
Dr. Youry Se, MD
Dr. Kurt Schaecher, PhD

b. Objectives:

1. To protect, project and sustain the military soldier against disease threats produced by the 2 major species of malaria, *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv). To support this mission through the evaluation of new or improved vaccines, prophylactic and therapeutic drugs, rapid diagnostic kits, and the maintenance of a center for excellence focused on the basic biology and epidemiology of malaria.

2. To assess emerging febrile diseases along high-risk regions in Thailand and throughout SE Asia

c. Methods:

The Department of Immunology and Medicine has applied as many kinds of classical and state-of-the-art technologies as possible to the above multi-faceted research. Clinical research included mobile epidemiology team able to work in adverse conditions where malaria is present, including field sample collection and processing screening, reference microscopy, assessment of rapid diagnostics for various tropical infectious diseases, and a staff well-versed in conduct of clinical trails to GCP and ICH standards. The animal research teams are all trained in laboratory animal research and regulations, current AALAAC requirements, and laboratory animal test and observation methods. State-of-the art methodologies are available for the studies of vaccine and

drugs to include advanced molecular biology methods such as sequencing and SNP analysis, and real-time pCR. Cellular immunology techniques are available which include flow cytometry and sorting technologies, ELISPOT and molecular methods. Pharmacology assays include HPLC, LC-MS, a unique malaria bioassay to measure the in vivo antimalarial bioactivity of potential new antimalarial medications, sustained malaria cell culture and radioisotopic uptake and antibody based methods for measuring in vitro drug sensitivity patterns of malaria strains against standard malaria drugs.

d. Results (accomplishments during the period of January 2004 - December 2004):

1. Malaria Vaccines STEP F/STO AF/STO A1

Continued efforts to develop a *P. vivax* human malaria challenge model. This model will allow better understanding of immunologic processes early in the development of Pv malaria, phase IIa pilot efficacy studies of candidate Pv vaccines, and testing of drugs with causal prophylactic and radical cure properties of Pv in a controlled setting. The AFRIMS' Departments of Immunology and Medicine and Entomology are uniquely situated to develop this strategic model. Efforts continuing in to FY05 to complete the development of the model. MIDRP funded.

Published results of a rhesus safety and immunogenicity protocol for a candidate Pf malaria multi-antigen, multi-stage vaccine. The vaccine involves combinations of RTS,S, $MSP1_{42,}$ and AMA-1 candidate vaccines, adjuvanted with our lead agent, AS02A. 46 rhesus monkeys were immunized over a 3-month schedule, and the vaccine is safe and well tolerated. Antibody and cellular immunology assays suggest immune competition may occur at the doses chosen. Further testing of two groups was approved and funded by USAID, and in-life portions are now completed. This MIDRP effort is partnered with GlaxoSmithKline and USAID.

Published result of study comparing the safety and immunogenicity of the adjuvant systems (AS01B, AS02A, AS05 and AS08) to alum in rhesus monkeys. Whereas, all MSP1(42) formulations tested generated high stimulation indices for lymphocyte proliferation (ranging from 27 to 50), the AS02A and AS01B formulations induced the highest levels of specific anti-MSP1(42) antibody. ELISPOT assays showed that the AS02A and AS01B vaccine formulations-induced different cytokine response profiles. Using the ratio of IFN-gamma/IL-5 secreting cells as the metric, the AS01B formulation induced a strong Th1 response, whereas the AS02A formulation induced a balanced Th1/Th2 response. The IFN-gamma response generated by AS02A and AS01B formulations persisted at least 24 weeks after final vaccination. The notable difference in Th1/Th2 polarization induced by the AS02A and AS01B formulations was notablerecombinant Plasmodium falciparum MSP1(42) antigen formulated with four novel

Published results of the malaria vaccine RTS,S combined with thrombospondin-related anonymous protein (TRAP) and formulated with AS02A (RTS,S+TRAP/AS02A). Here, RTS,S+TRAP/AS02A was administered on a 0-, 1-, and 3-month schedule to three cohorts of infant monkeys, along with adult comparators. Cohort 1 evaluated 1/5, 1/2, and full adult doses,

with the first dose administration at one month of age; cohort 2 monkeys received full adult doses, with the first dose administration at one versus three months of age; and, cohort 3 compared infants gestated in mothers with or without previous RTS,S/AS02A immunization. Immunization site reactogenicity was mild. Some infants, including the phosphate-buffered saline only recipient, developed transient iron-deficiency anemia, which is considered a result of repeated phlebotomies. All RTS,S+TRAP/AS02A regimens induced vigorous antibody responses that persisted through 12 weeks after the last vaccine dose. Modest lymphoproliferative and ELISPOT (interferon-gamma and interleukin-5) responses, particularly to TRAP, approximated adult comparators. RTS,S+TRAP/AS02A was safe and well tolerated. Vigorous antibody production and modest, selective cell-mediated immune responses suggest that RTS,S+TRAP/AS02A may be immunogenic in human infants.

Published results of a study showing that blood stage schizonts or soluble schizont extracts activated plasmacytoid dendritic cells (PDCs) to up-regulate CD86 expression and produce IFN-alpha. IFN-alpha production was also detected in malaria-infected patients, but the levels of circulating PDCs were markedly reduced, possibly because of schizont-stimulated up-regulation of CCR7, which is critical for PDC migration. The schizont-stimulated PDCs elicited a poor T cell response, but promoted gamma delta T cell proliferation and IFN-gamma production. The schizont immune stimulatory effects could be reproduced using murine DCs and required the Toll-like receptor 9 (TLR9)-MyD88 signaling pathway. Although the only known TLR9 ligand is CpG motifs in pathogen DNA, the activity of the soluble schizont extract was far greater than that of schizont DNA, and it was heat labile and precipitable with ammonium sulfate, unlike the activity of bacterial DNA. These results demonstrate that schizont extracts contain a novel and previously unknown ligand for TLR9 and suggest that the stimulatory effects of this ligand on PDCs may play a key role in immunoregulation and immunopathogenesis of human falciparum malaria.

Two preclinical vaccinology studies are planned for FY 2005:

A safety and immunogenicity study of a prime boost strategy for SIV gag protein in rhesus monkeys using prime boost strategies with an adenovirus construct continues to be executed in FY05. This technology, which promises enhanced CD-8 T cell cellular responses may have utility in future malaria vaccine designs. Due to start in March 2004.

Testing safety and immunogenicity of LSA-1 vaccine constructs in combination with RTSS in rhesus monkeys is planned for spring 2005. MIDRP funded.

Malaria Drugs STEP Q, STO-AQ, STO-A4, STO-A5

Published methods paper describing a simple, nonisotopic, semiautomated bioassay for the measurement of antimalarial drug levels in plasma or serum based on the quantitation of histidine-rich protein II in malaria culture is presented. The assay requires only small sample volumes and was found to be highly sensitive and reproducible. The results closely

paralleled those obtained with isotopic bioassays ($R = 0.988$, $P < 0.001$) and high-performance liquid chromatography-electrochemical detection ($R = 0.978$, $P < 0.001$).

Published results of a histidine-rich protein 2 (HRP2)-based drug sensitivity assay for testing of fresh isolates of Plasmodium falciparum in the field. In contrast to the HRP2 laboratory assay, the field assay uses a procedure that further simplifies the handling and culturing of malaria parasites by omitting centrifugation, washing, the use of serum, and dilution with uninfected red blood cells. A total of 40 fresh Plasmodium falciparum isolates were successfully tested for their susceptibility to dihydroartemisinin, mefloquine, quinine, and chloroquine (50% inhibitory concentration [IC50] = 3.43, 61.89, 326.75, and 185.31 nM, respectively). Results very closely matched those obtained with a modified World Health Organization schizont maturation assay ($R2 = 0.96$, $P < 0.001$; mean log difference at IC50 = 0.054).

Added liquid chromatography-mass spectrometry capabilities at AFRIMS for the primary purpose of high-throughput GLP testing of artesunate and its metabolites in the clinical development program. GLP validation in progress for planned Phase I and 2 human studies in FY05. MIDRP and MMV funded.

Based on in vitro and rodent malaria data, a new study of the efficacy of IV artesunate and methylene blue combinations against *P. cynomolgi* malaria in rhesus was initiated. In life completed in mid 2004, and PK curves of methylene blue in rhesus completed. PK data reported at 2004 ASMTH and JITTM meetings. ILIR funded.

Secured funding and developed a new Phase II dose ranging protocol of azithromycin/quinine and azithromycin/artesunate combinations for the treatment of uncomplicated falciparum malaria. In life portion currently in progress in collaboration with the Hospital of Tropical Diseases, Mahidol University. Efforts are partnered with Pfizer and the NIH.

Executed clinical trial of tafenoquine monotherapy in adults for evaluation of radical curative ability and pharmacokinetics in *P. vivax* malaria. This study was fully successful in demonstrating that tafenoquine monotherapy can effectively eliminate both blood and liver stage parasites in a manner compatible with current therapies. Funded with NIH co-development grant with GSK, and partnered with Hospital of Tropical Diseases, Faculty of Tropical Medicine, Mahidol University. Data analysis if currently in progress with publication anticipated in mid 2005.

Published results of a tafenoquine dose ranging clinical trial to compare tafenoquine with primaquine for the radical cure (hypnozoite eradication) of P. vivax, 80 patients with P. vivax infection were randomized to receive 1 of the following 5 treatments 1 day after receiving a blood schizonticidal dose of chloroquine: (A) tafenoquine, 300 mg per day for 7 days (n=18); (B) tafenoquine, 600 mg per day for 3 days (n=19); (C) tafenoquine, 600 mg as a single dose (n=18); (D) no further treatment (n=13); or (E) primaquine base, 15 mg per day for 14 days

(n=12). The minimum duration of protocol follow-up was 8 weeks, with additional follow-up to 24 weeks. RESULTS: Forty-six of 55 tafenoquine recipients, 10 of 13 recipients of chloroquine only, and 12 of 12 recipients of chloroquine plus primaquine completed at least 8 weeks of follow-up (or had relapse). There was 1 relapse among recipients of chloroquine plus tafenoquine, 8 among recipients of chloroquine only, and 3 among recipients of chloroquine plus primaquine. The rate of protective efficacy (determined on the basis of reduction in incidence density) for all recipients of chloroquine plus tafenoquine, compared with recipients of chloroquine plus primaquine, was 92.6% (95% confidence interval, 7.3%-99.9%; P=.042, by Fisher's exact test). CONCLUSIONS: Tafenoquine doses as low as a single 600-mg dose may be useful for prevention of relapse of P. vivax malaria in Thailand.

Published study assessing monthly doses of tafenoquine for preventing Plasmodium vivax and multidrug-resistant P. falciparum malaria. In a randomized, double-blind, placebo-controlled study, 205 Thai soldiers received either a loading dose of tafenoquine 400 mg (base) daily for 3 days, followed by single monthly 400-mg doses (n = 104), or placebo (n = 101), for up to 5 consecutive months. In volunteers completing follow-up (96 tafenoquine and 91 placebo recipients), there were 22 P. vivax, 8 P. falciparum, and 1 mixed infection. All infections except 1 P. vivax occurred in placebo recipients, giving tafenoquine a protective efficacy of 97% for all malaria (95% confidence interval [CI], 82%-99%), 96% for P. vivax malaria (95% CI, 76%-99%), and 100% for P. falciparum malaria (95% CI, 60%-100%). Monthly tafenoquine was safe, well tolerated, and highly effective in preventing P. vivax and multidrug-resistant P. falciparum malaria in Thai soldiers during 6 months of prophylaxis.

Supported parasitology requirements for continuing efforts to develop a hepatocyte cell line to screen activity in the liver of antimalarial drugs and vaccine candidates. Collaboration with Department of Entomology. MIDRP funded.

Diagnostics/Rapid Diagnosis of Malaria STEP-L/STO-L

Continued development of real-time pcr method to reliably diagnose Pv and Pf malaria from human blood. Assessed performance characteristics of assay with attention focused in low parasitemias, where traditional microscopic diagnosis is prone to error.

Continued evaluation of available scrub typhus tests to develop a gold standard for comparison to immunochromatographic scrub typhus (*Orientia tsutsugamushi*) rapid diagnostic tests in development.

Tested two commercially available leptospirosis diagnostic tests at the Sangkhlaburi febrile diseases study site for preliminary assessment by the STO L committee. Analysis in progress.

Emerging Infectious Diseases (GEIS)

Epidemiology of Falciparum Malaria Drug Resistance Patterns in Asia:

Continued surveillance activities throughout Southeast Asia (Bangladesh, Myanmar, Thailand and Vietnam) for threat assessment of multi-drug resistant malaria. Assessed potential new field sites in Cambodia, eastern Bangladesh, Nepal and northern Thailand (Chiang Dao). Parasite isolates continue to analyzed longitudinally to assess for trends in antimalarial drug sensitivity patterns potentially signaling a dimunition in the utility of the present armamentarium of malaria medications. GEIS funded, and coordinated with Public Health departments in the various countries.

Validated under field conditions a new non-isotopic method for *in vitro* drug resistance assays, which is simpler, as robust, and avoids radioisotopes. The methods has been made available free of charge to the malaria research community as a public service (see http:/malaria.farch.net). The test shows very reliable comparisons to the WHO microtest using a much simpler methodology. Funded by GEIS with support from Mahidol University and University of Vienna.

Surveillance of Febrile Diseases along the Thai-Myanmar Border:

Continued a multi-year effort to establish infectious etiologies to undifferentiated fevers along the Thai-Myanmar border in Kanchanaburi province. Over 1000 persons enrolled. Malaria accounts for approximately 25% of adults with fever. Leptospirosis appears to be a frequent, but previously unrecognized cause of morbidity and mortality, as is spotted fever rickettsia. Our data has led to a change to local health treatments. Conducted a province-wide health education seminar attended by over 70 local health care personnel aimed at reporting the results of the fever study in order to promote a greater understanding and improved, evidence-based treatment algorithms for local health care providers.

e. Future plans:

We plan to continue our multi-faceted emphasis on support for malaria product development in diagnostics, new drugs, and new vaccines. We anticipate heavy participation in a DoD wide effort on malaria microscopy QA procedures and have hired a dedicated expert teaching microscopist charged with the responsibility of developing a rigorous microscopy teaching and certification standard. Furthermore, we anticipate being the lead overseas lab for field-testing intravenous artesunate in phase I and II, as possibly phase III testing. We will continue efforts for tafenoquine development, especially towards an indication of radical cure for *Plasmodium vivax*. We will continue safety and immunogenicity testing of candidate malaria vaccines in rhesus, and progress towards vivax challenge studies for eventual human testing of vivax vaccines in Thailand. Emerging infection work in Sangkhlaburi will continue with emphasis on flaviviruses, leptospirosis and typhoidal illnesses, and this study will be expanded to another targeted site in Nepal. Lastly, we anticipate an expand role in regional malaria surveillance with a combination of in vivo, in vitro and genetic methods to define expanding malaria drug resistance.

C. Department of Enteric Diseases, AFRIMS FY04 Research Efforts.

1. Title of research project: Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand

a. Investigator:

Dr. Ladaporn Bodhidatta

b. Objectives:

Monitor diarrhea etiology and antimicrobial resistance of enteric pathogens at multiple sites within Thailand.

c. Methods:

Hospitals and regional laboratories in several sites in Thailand to include Trang, Mae Hong Son, Udon Ratchathoni, Samut Sakhon, Bangkok, and other locations agreed to participate. The study protocol was approved by both the US and Thai authorities. Several visits were made to each site by the Principal Investigator, as well as nursing and laboratory staff prior to study initiation. Stool samples were received at each participating site for initial assessment followed by additional laboratory studies to include culture and sensitivity at AFRIMS in Bangkok Thailand.

d. Results:

Over 100 stool samples have been received.

e. Future plans:

Continue study.

2. Title of research project: Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

a.. Investigators:

Orntipa Sethabutr
Rungnapha Phasuk
Sasikorn Silapong

b. Objectives:

Develop and standardize realtime PCR assays for the detection and characterization of enteric pathogens to include Shigella, Salmonella, Campylobacter, Cryptosporidia, Cyclospora, and Noroviruses.

c. Methods:

Based on literature review and best available sequence data, multiples sets of primers and probes were designed for each pathogen of interest. The sets were initially evaluated against cultured material. Selected sets of primers and probes were then tested against frozen stool samples collected and archived from multiple Department of Enteric Diseases studies. Lower limits of detection for several sets of primers and probes were determined.

d. Results:

Probes and primer sets have been developed for Campylobacter, Shigella, Salmonella, Cryptosporidia, Cyclospora, and Noroviruses.

e. Future plans:

Continue study.

3. Title of research project: Travelers' Diarrhea among US Forces Deployed for Operation Cobra Gold

a. **Investigator:**

Dr. Bryan Smith

b. **Objectives:**

Determine diarrhea etiology and antimicrobial resistance of enteric pathogens from diarrhea from US soldiers deployed to two different sites within Thailand. Study the Campylobacter specific immune responses in cases with diarrhea and assymptomatic controls.

c. Methods:

A human use protocol describing the study was submitted for US human use approval. Field laboratories were set up during Operation Cobra Gold 2004 at two sites in Thailand. After obtaining informed consent, stool and blood specimens were collected from diarrhea cases on presentation and five and ten days later (days 0, 5, and 10). An effort was made to obtain a matched assymptomatic control for each case.

d. Results:

The number of diarrhea cases during Operation Cobra Gold continued to decline. Collecting specimens on a fixed schedule during a short exercise was difficult and few complete sets of specimens were obtained. The predominant pathogens were Campylobacter and Salmonella.

e. Future plans:

Conduct etiology study during Operation Cobra Gold 2005 at one site. Investigate other military populations with great risk of diarrhea for future studies.

4. Title of research project: Characterization of Enteric Pathogens Isolated from Children in Phnom Penh

a. Investigators:

Dr. Bryan Smith
Dr. Ladaporn Bodhidatta

b. Objectives:

Determine diarrhea etiology and antimicrobial resistance of enteric pathogens from young children with diarrhea presenting to the National Pediatric Hospital in Phnom Penh, Cambodia.

c. Methods:

A human use protocol describing the study was submitted for US and Cambodian human use approval. Additional equipment and supplies were ordered for the microbiology laboratory at the National Pediatric Hospital in Phnom Penh. After obtaining informed consent, stool specimens were collected from diarrhea cases on presentation. An effort was made to obtain a matched assymptomatic control for each case. Initial stool examination and culture was performed at the National Pediatric Hospital. Additional studies to include antimicrobial susceptibility testing were conducted at AFRIMS.

d. Results:

Common diarrheal pathogens identified thus far include Rotavirus and Shigella.

e. Future plans:

Continue study for a total of two years.

D. Department of Veterinary Medicine AFRIMS FY04 Research Accomplishments

1. Title of research project: Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta)/Plasmodium cynomolgi* Relapsing Malaria Model.

a. Investigator:

Dr. Montip Gettayacamin

b. Objectives:

1) Use the rhesus monkey/*P. cynomolgi* model to determine the effectiveness of new causal prophylactic and radical curative compounds which are being synthesized and developed by the US Army antimalarial drug development program.

2) Us the rhesus monkey/*P. cynomolgi* blood-stage malaria model to evaluate new antimalarial compounds for their blood schizonticidal activity.

c Methods:

Malaria is one of the most important parasitic diseases worldwide. Traditional treatment for malaria includes drugs used to prevent disease (prophylaxis) and to cure the infection (therapeutic). Antimalarial drug screening in the rhesus monkey model is very effective for making comparisons between drugs. It is fairly rapid, relatively inexpensive, and makes reliable predictions of how drugs will in act in man. Antimalarial drug screening in the rhesus monkey has played a key role in the development of every antimalarial drug licensed in the the US for the past 30 years. This model provides a mechanism to identify effective new drugs for the enhanced prevention and treatment of malaria infections.

d. Results:

The protocol successfully tested 2 new potential antimalarial compounds for causal prophylactic and radical curative activity.

e. Future plans:

We anticipate testing at least two new compounds over the next calender year.

2. Title of research project: Care and Maintenance of Rhesus *(Macaca mulatta)* and *Cynomolgus (Macaca fascicularis)* Monkeys and Management of Breeding Colonies

a. Investigators:

Dr. Montip Gettayacamin
Mr. Srawuth Komcharoen

b. Objectives:

Maximize the production of specific pathogen-free rhesus and cynomolgus monkeys in the USAMC-AFRIMS production colony, using the best and most humane husbandry care, maintenance procedures, veterinary care, and disease surveillance and environmental enrichment procedures available.

c. Methods:

USAMC-AFRIMS maintains a breeding colony of rhesus and cynomolgus macaques using a closed colony system. Approximately 250 rhesus and 50 cynomolgus monkeys are used in the breeding program. Two types of breeding is managed: compatible male and female pairs are housed in special paired-type caging, and multiple harem groups are established and maintained in large gang cages. Harems consist of one breeding sire and 5-15 adult females. Newborn monkeys are weaned at approximately 6 months of age, and then are reared to adulthood in gang cages with other weanlings. All colony primates are tested routinely for the presence of infectious diseases that pose a threat to either the health of the colony or to personnel working with the primates. Humane use of the animals is assured by the intense oversight of the Institutioanl Animal Care and Use Committee. Veterinary and technical care is extensive and continuous.

Whenever possible, animals are re-utilized in multiple protocols in order to optimize the use of this limited and essential resource.

e. Results:

Approximately 57 rhesus and 3 cynomolgus macaques were produced in the previous year.

f. Future plans:

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free nonhuman primates to support USAMC-AFRIMS research needs. Maintain and expand the colony by obtaining 20 new breeding males, increasing the number of paired housing cages, and placing breeding pairs in these new cages into additional animal rooms in the vivarium.

3. Title of research project: Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance / Quality Surveillance Program

a. Investigators:

Dr. Montip Gettayacamin
Ms. Anchalee Tungtaeng

b. Objectives:

Maintain a breeding colony of specific pathogen-free laboratory rodents to meet the scientific research needs of the USAMC-AFRIMS, using state-of-the-art knowledge, equipment, and facilities.

c. Methods:

USAMC-AFRIMS maintains breeding colonies of laboratory rodents to meet the needs of AFRIMS research. Using state-of-the-art equipment, knowledge, and facilities, production is matched to the anticipated needs of individual research projects. Extensive and thorough recordkeeping ensures that outbred strains remain outbred, and that inbred strains remain truly inbred. An extensive quality assurance/quality surveillance program, which includes serologic assessments as well as necropsy/histopathologic analysis, ensures that the colony produces only high-quality disease-free animals. When necessary, new breeder stock is procured from a reliable vendor in the United States or Japan. Veterinary and technical care is extensive and continuous.

d. Results

One thousand five hundred eighteen (1518) litters of ICR mice (*Mus Musculus*) were produced, comprising a total of 19,175 mouse pups. Quality surveillance evaluations were carried out using 15 ICR mice. Quality assurance was performed in 10 hamsters purchased from the National Laboratory Animal Center.

e. Future plans:

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free rodents to support USAMC-AFRIMS research needs.

4. Title of research project: A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs

a. Investigators:

Dr. Montip Gettayacamin
Pranee Hansukjariya
Anchalee Tungtaeng

b Objectives:

To evaluate potential antimalarial chemotherapeutic agents in the *P. berghei* ICR mouse - the modified Thompson Test model.

c. Methods:

The test system used for the determination of antimalarial activity of the compounds is a modification of the suppressive test known as the Thompson Test. Typically in this test, up to 22 groups of 8 mice are inoculated intraperitoneally (IP) with *P. berghei*-infected erythrocytes then treated with candidate drugs to determine the antimalarial activity. Infected erythrocytes are provided from donor mice. On experiment day 0, the donor mice are anesthetized then exsanguinated via cardiac puncture, the blood pooled and the level of parasitemia determined. The pooled blood is then diluted with normal mouse serum to a concentration of 1×10^6 P. *berghei*-infected erythrocytes per inoculum (0.1 ml). The groups of experimental and control mice are inoculated with this parasitized blood on day 0. On day 3, 4, and 5 mice are treated with either the candidate antimalarial drug or with vehicle alone, to serve as the negative control. The drug is administered orally (PO), subcutaneously (SC), intramuscularly (IM), and/or intraperitoneally (IP) up to three times a day, based on the individual and unique pharmacodynamics of the test compound. Each experimental group receives a different dose level, with up to 7 different dose groups per compound. A standard antimalarial drug may be tested along with the candidate drug for structure-activity determination and for quality assurance of the model. Blood films and body weights are taken on the third and sixth days post-infection, then at weekly intervals though day 60. Blood films are stained, examined by light microscopy, and the percent parasitemia determined. All mice are observed twice a day to assess their clinical signs. All mice with negative smears at 60 days are considered cured.

d. Results:

A total of 20 compounds were tested in 9 experiments.

e. Future plans:

This mouse model for screening new candidate antimalarial compounds has been used for over 30 years and is very effective for making comparisons between drugs. It is rapid, relatively inexpensive, and makes reliable predictions of how drugs will act in higher mammalian hosts, including humans. This is a core capability of the USAMC-AFRIMS Department of Veterinary Medicine and will be maintained so that many more compounds can be tested.

5. Title of research project: Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelli*) for Screening Exoerythrocytic Antimalarial Drugs

a. Investigators:

Dr. Montip Gettayacamin
Dr. Jetsumon Prachumsri, Ph.D.
Anchalee Tungtaeng
Dr. Robert S. Miller
Dr. Dennis Kyle

c. Objectives:

To evaluate potential causal prophylactic antimalarial agents in the P. yoelii mouse exoerythrocytic (EE) model at AFRIMS.

d. Methods:

A model involves infecting mice with sporozoites harvested from infected Anopheline dirus mosquitoes on day 0. The infected mice are dosed with test compound on day -1, prior to inoculation of sporozoites and then on day 1. There are 6-16 groups of 5 mice or up to 80 mice in each experiment. The routine test consists of 1 to 3 dosage levels of up to 5 compounds administered by one or two routes. One group will receive vehicle alone to serve as the control. Blood films, weight and clinical signs are followed to 30 days post-infection. Mice with negative smears at 30 days are considered to be protected.

d. Results:

The model is available for use when candidate compounds are ready for testing.

e. Future plans:

We expect to screen approximately up to 100 compounds in 20 experiments (5 compounds at 3 dosages, 16 groups or 80 mice per experiment) in FY05. However, the selection of drugs to be tested, the dosing regimens to be used, and the prioritization of the experiments are the responsibility of the WRAIR-ET.

E. Department of Virology, AFRIMSFY04 Research Accomplishments

1. Title of research project: The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue

a. Investigators:

1. Principal Investigators:

Siripen Kalayanarooj, MD (Queen Sirikit Institute of Child Health, Bangkok)
Stephen J. Thomas, MAJ, MC (USAMC-AFRIMS)

2. Associate Investigators:

Mammen P. Mammen, Jr. LTC, MC (USAMC-AFRIMS)
Chunlin Zhang, DVM, PhD, MAJ, MS (USAMC-AFRIMS)
Chuanpis Ajariyakhajorn, DVM, Research Coordinator (AFRIMS)
Ananda Nisalak, MD (AFRIMS)
Pra-orn Supradish, MD (QSNICH)
Anchalee Krautrachue, MD (QSNICH)
Lawan Wongtapradit, MD (QSNICH)
Narong Nithipanya, MD (QSNICH)
Warangkana Ratanaprakarn, MD (QSNICH)
Anon Srikiatkhachorn, MD, Assistant Professor (UMMS)
Daniel H. Libraty, MD, Assistant Professor (UMMS)
Irene Bosch, PhD, Assistant Professor (UMMS)
Alan L. Rothman, MD, Associate Professor, (UMMS)
Sharone Green, MD, Associate Professor, (UMMS)
Francis A. Ennis, MD, Director (UMMS)
Henry A. F. Stephens, Ph.D., Clinical Scientist and Head of Tissue Typing (University College London)

b. Objectives:

This study continues to investigate pathophysiologic mechanisms of illness resulting from dengue infections. Information gained from this study provides important insight into the methods of preventing and intervening in severe dengue disease. The project encompasses studies from 2003 to 2007.

c. Study Specific Objectives:

1. Characterize genetically and functionally the dengue virus-specific T lymphocyte response during, and after dengue virus infections (intracellular cytokine staining, HLA tetramers, T cell receptor gene usage);

2. Analyze interactions between dengue virus, virus-specific antibodies, and target cells in PBMC during acute dengue virus infections (quantify and characterize immune complexes, define the major cellular compartments in PBMC supporting dengue viral replication);

3. Determine if ultrasound or interstitial fluid albumin levels can predict early plasma leakage and shock. The ability to detect these shifts early in disease progression may help in prediction algorithms for DHF and permit early intervention with new therapies in the at-risk population

4. Assess the utility of plasma sNS1 levels in predicting disease severity for subjects with primary or secondary infection due to any of the four dengue serotypes

5. Analysis of the activation of innate immune responses in vivo during acute dengue virus infections (chemokine gene expression, inhibitory and activating NK receptor expression);

6. Identification of polymorphisms in immune response genes associated with disease manifestations and cellular immune responses during dengue virus infections (MHC class I and II, Fcγ receptor gene, KIR genes, NK receptors) and MHC class I chain-related (MIC) genes (ligands for lectin-like receptors);

7. Quantitation of viral burden in plasma and cell subsets of peripheral blood mononuclear cells (PBMC) for all four serotypes in primary and secondary dengue virus infections and determine if there is a correlation between viral load in these compartments and disease severity;

8. Measurement of neutralizing antibody elicited by primary infections, over an extended period of time. Few long-term studies of antibody titer following dengue infection have been performed previously. Neutralizing antibody will be measured on study day 1, 6 months, 1 year, and annually thereafter. Understanding wild type responses will help to set realistic standards for vaccines. Mature secondary responses determined by neutralization six months or more after infection will be correlated with class II HLA type;

9. Determination of memory T-cell responses following primary and secondary dengue infections, over an extended period of time Understanding wild type responses and the durability of these responses over time will be crucial in setting standards for testing of candidate dengue vaccines;

10. Continue sequencing portions of the dengue genome from patients with mild dengue fever and those with severe DHF/DSS to test a hypothesis that severity of disease is strain related. In addition, compare the kinetics of plasma viral load and immune responses in primary and secondary infections with different DV serotypes;

11. Evaluate the accuracy of sequentially measured semi-quantitative d-dimer assay, as compared to standard clinical parameters, at predicting the clinical progression to severe clinical dengue.

d. Methods:

Children were enrolled if they were suspected of having an early DV infection (without evidence of DHF) or a fever without an identifiable source. Inclusion criteria included an oral temperature $\geq 38.5°C$, fever onset not longer than 72 hours prior to the initial evaluation,

weight > 6kg, flushed face, signed consent by parent or guardian. After informed consent is obtained, subjects are admitted to the hospital and a blood specimen obtained. The result of the plasma test for DV RNA by RT-PCR is available the morning of study day 2. Children who are DV RT-PCR-negative are given the opportunity to leave the study, or to continue in the study for clinical observation. Those children remaining in the hospital undergo inpatient observation until one day following defervescence (fever day +1). Clinical information is collected and recorded daily. Radiographic studies are performed as outlined in the protocol. Serial blood samples are collected and analyzed for routine and dengue-specific blood and plasma tests were conducted to include, but not limited to:

1. CBC, WBC differential, AST, Albumin
2. Hemagglutination inhibition (HAI) assay for dengue
3. Antibody-capture DV IgM/IgG enzyme immunoassay (EIA)
4. RT-PCR for dengue, Plasma viremia titers
5. Dengue virus isolation in Toxorhynchites splendens and typing
6. IL-15, IL-18, MIP-1a, MIP-1b, and MCP-1, CD69, CD38, and Ki-67
7. Labeled antibodies to identify T cell subsets, NK cells and B cells
8. NS1 (soluble NS1 and anti-NS1 antibodies)
9. Complement assays

f. Results:

We enrolled 97 subjects from 11 May to 30 Nov 2004. There were 50 positive PCR cases (DEN 1 = 21; DEN 2 = 5; DEN 3 = 1; DEN 4 = 23; Negative PCR = 47). Alls subjects had ultrasound evaluation for plasma leakage. A subset of dengue positive cases had interstitial fluid sampling done. Three cases were lost to follow-up (all are non-dengue diagnoses). One case moved to north-east Thailand and is unavailable for follow-up. No serious adverse events occurred.

g. Future plans:

Long-term clinical follow-up is ongoing for prior years of enrollment and we are preparing for enrollment next year. Analysis for markers that predict disease severity (d-dimer, NS1 protein/antibody levels, immune activation markers), that indicate plasma leakage is or will occur, and that indicate immunity will be done. Statistical analysis of DHF resulting from primary versus secondary DV infections with regard to the role viral serotype, viral burden and virus-antibody complexes plays on resulting disease severity is planned. Characterization of the dengue specific T cell response with regard to the magnitude of T cell expansion during infection and the functional characteristics of these cells is also planned.

2. Title of research project: A Recombinant Hepatitis E Vaccine Efficacy Study in Nepalese Volunteers

a. Investigators:

1. Principal Investigators:

   M. P. Shrestha (WARUN)
   R. M. Scott (WARUN)

2. Associate Investigators:

   G. B. Thapa (SBH)
   K. S. A. Myint (AFRIMS)
   R. A. Kuschner (WRAIR)
   D. M. Joshi (SBH)
   M. P. Mammen (AFRIMS)
   B. L. Innis (GSK)
   S.K. Shrestha (WARUN)

b. Objectives:

To evaluate the protective efficacy for the prevention of hepatitis E disease provided by the candidate hepatitis E vaccine administered according to a 0, 1 month schedule with a booster dose at month 6.

c. Methods:

A candidate recombinant baculovirus expressed hepatitis E virus (HEV) vaccine was found to be safe and immunogenic in 88 American and 44 Nepali volunteers. A 20μg formulation was selected for further evaluation in a randomized double blind placebo controlled efficacy trial in susceptible, active duty Royal Nepal Army volunteers. Of 5,571 consenting volunteers screened, 3,113 were susceptible to HEV. Two thousand volunteers (5 females, 1,995 males) were enrolled, receiving either placebo or 20μg of active vaccine. Volunteers were vaccinated at 0, 1, and 6 months with sera collected at months 0, 1, 3, 6, 7, 13, and 24. One tenth of the volunteers were followed on days 1, 3, 5, and 7 after each vaccination for local and general solicited adverse events (SoAE). Non-serious adverse events (NSAE) were recorded for 30 days after each vaccination and serious adverse events (SAE) were to be collected throughout the 2-year study period. Sera and stool from cases meeting clinical and biochemical criteria compatible with viral hepatitis, were examined for HEV RNA by a reverse transcriptase-polymerase chain reaction, and serologically for HEV IgM and Ig, HAV IgM, HBsAg, HBcIgM and HCV IgG.

One thousand eight hundred and twenty five (1,825) volunteers received a three vaccine series. >86 HEV cases occurred during the two year period. Clinical phase closure was on 16 DEC 03 and initial unblinding of 111 cases of hepatitis was conducted on 23 JUN 04.

d. Future plans:

Formal release of unblinding information is forthcoming for possible further development and testing of this vaccine.

3. Title of research project: Prospective Study of Dengue Virus Transmission and Disease in Primary Schools and Villages in Kamphaeng Phet, Thailand

a. Investigators:

1. Principal Investigators:
   Suwich Thampolo, MD, MPH
   Dengue Office, Division of Vector-Borne Diseases
   Ministry of Public Health (MOPH)

   Mammen P. Mammen Jr, LTC, MD, MC (USAMC-AFRIMS)

2. Associate Investigators (by institution):

Armed Forces Research Institute of Medical Science (AFRIMS):

Department of Virology

1. Ananda Nisalak, M.D., Consultant in Arbovirology
2. Stephen Thomas, M.D., MAJ, MC, Assistant Chief and Head of Field Operations
3. Chunlin Zhang, DVM, Ph.D.,MAJ, MS, Head, Laboratory Operations
4. Chuanpis Ajariyakhajorn, DVM, Research Coordinator

Department of Entomology

1. James Jones, Ph.D., MAJ, MSC, USA, Departmental Chief
2. Ratana Sithriprasana, Ph.D. Candidate and Head, Mosquito Biology Section

Thai Ministry of Public Health (MOPH):

1. Virat Puthimethee, M.D., Office of the Provincial Public Health, KPP
2. Supamit Chunsuttiwat M.D., Senior Medical Officer, Ministry of Public Health
3. Somsak Prajakwong, M.D., Director of Vector-borne Disease Control Office

Institute of Urology and Nephrology, University College London, The Middlesex Hospital,

1. Henry A. F. Stephens, Ph.D., Clinical Scientist and Head of Tissue Typing

Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School (UMMS):

1. Anon Srikiatkhachorn, M.D., Assistant Professor, e-mail: anons@thai.amedd.army.mil
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5. Francis A. Ennis, M.D., Director, e-mail: francis.ennis@umassmed.edu .

Department of Entomology, University of California, Davis:

1. Thomas W. Scott, Ph.D., Professor of Entomology and Director, Davis Arbovirus Research Unit
2. John D. Edman, Ph.D., Professor of Entomology and Director, Center for Vector-Borne Diseases
3. Amy C. Morrison, Ph.D., Assistant Research Entomologist

Department of Geography, San Diego State University:

1. Arthur Getis, Ph.D., Stephen and Mary Birch Chair of Geographical Studies

b. Objectives:

The goal of the proposed study is to identify those factors that have the strongest influence on determining the early events in acute DV infections, and the eventual clinical manifestations of disease. An equally important goal is to characterize protective immune responses (e.g. $CD4^+$ and $CD8^+$ T-cell responses, neutralizing antibody responses) as we have found that low levels of pre-existing neutralizing antibodies to a subject's own infecting virus isolate do not necessarily protect from symptomatic DV infection. We plan to prospectively identify host-specific factors (e.g. pre-existing memory T and B cell responses to DV, HLA genetic polymorphisms, viral burden and replication in the host), virus-specific factors (e.g. DV serotype, serotype infection sequence), and environmental factors (e.g. mosquito population patterns, mosquito viral burden) for asymptomatic and symptomatic secondary DV infections, particularly severe infections (DHF/DSS). Multi-year investigations are crucial to this study due to the year-to-year variations in the incidence and prevalence of circulating serotypes. An improved understanding of the correlations between the host, viral, and environmental factors and dengue disease severity will contribute to DV vaccine development and testing.

c. Study Specific Hypotheses:

1. Subjects with pre-existing neutralizing dengue antibodies above a definable threshold will be protected from DV infection or severe disease on subsequent exposure to virus.

2. The frequency of pre-existing $CD4^+$ and $CD8^+$ T-cells and their specific cytokine responses to stimulation with DV antigens will correlate with disease severity (protection or enhancement) and the plasma viral RNA levels measured in secondary DV infections.

3. Specific serotype sequence combinations of DV infections will elicit qualitatively and quantitatively distinct immune responses associated with illness of varying severity.

4. Higher viremia levels will be seen in secondary DEN-2 and DEN-4 virus infections in subjects with higher levels of *in vitro* antibody-dependent enhancing capability of pre-illness blood samples.

5. DV infection rates will cluster in households around a DV-infected index case and a correlation will exist between the number of susceptible contacts, and associated mosquito density, and mosquito infectivity (viral RNA levels).

6. DV disease severity will correlate with peak plasma viremia levels and associated mosquito density and mosquito infectivity (viral RNA levels).

7. Genes encoded within the human MHC, the NK killer inhibitory receptor (KIR) gene complex on chromosome 19, and the Fc gamma receptor gene complex on chromosome 1 influence the susceptibility, severity and resistance to primary and secondary DV infections.

d. Methods:

In this study, we

i) Continue the successful prospective, school-based, study platform to study dengue epidemiology in primary school children in KPP province, and
ii) Add a village-based, cluster surveillance study.

a) This is a prospective school-based study of 2,000 children, which will begin upon study approval in 2003 and end in January 2008. Students in K2 to grade 6 will be recruited and enrolled into the study in June-July 2003 or upon study approval. Baseline demographics will be recorded and study numbers assigned. Each subsequent year, new K1-Grade 5 students will be newly enrolled. Students will be followed until they are either disenrolled, withdrawn by their parent/guardian, graduate from Grade 6 or when the study ends. Every year, plasma (plasma and PBMCs for Dengue Season 1 only) will be collected from the entire cohort at the beginning of the surveillance period (June). Plasma and PBMC will then be collected from the entire cohort at the end of the surveillance period (January). A one-dilution neutralization assay will be performed on paired sera from the beginning and end of the surveillance period to assess for flavivirus seroconversion. Plasma and PBMCs obtained at the end of the surveillance period in January will serve as pre-illness samples in subjects who have a DV infection that same calendar year.

During the active surveillance period extending between June and the following January, those children who are absent from school (or who report ill to the teacher), will be evaluated either by a VHW or AFRIMS nurse using a questionnaire and oral temperature measurement. Any child who has a documented fever (temperature $\geq$ 38C) or reports illness with subjective fevers during the prior 7 days, will be transported to the PHO where a public health nurse will do an evaluation. An acute blood specimen will be drawn. The child will be referred to hospital at the discretion of the public health nurse. About 14 days later, an AFRIMS nurse will visit the child to administer another questionnaire and to draw a convalescent blood specimen. The acute and convalenscent specimens will be evaluated by dengue/JE ELISA and HAI. The acute specimen will be evaluated further by RT-PCR (and virus isolation techniques).

b) Cases 'triggering' a cluster investigation will be identified between Monday and Thursday of each week during the School-Based Component active surveillance period. Most specimens from acutely ill children will arrive at the field station laboratory by 3pm each day. Upon arrival of the specimen, the database will be reviewed to assess whether the child meets all index case inclusion and exclusion criteria. The field teams will be notified of a possible case. The DV RT-PCR result (positive or negative) will normally be available by 11AM the following morning. No more than 30 positive and 30 negative clusters (as defined by the RT-PCR result of the index case) will be initiated in any given year. Once triggered, an Advance Team composed of a nurse and an entomological team supervisor will visit the village and begin the consent form process. The exact location of all houses in each participating village will have been previously determined using a Global Positioning System (GPS) unit. Data points will be used to construct a digital map which will enable the team to precisely identify houses located within 100-200 meter radius (the exact radius to be pre-determined based on the prevalent average density of homes across all villages) of the index case and rapidly assess the likelihood of enrolling a minimum of 10 contacts. Once at least 10 contacts have been consented, the field teams will be dispatched to the village where the consent form process will continue. A reasonable effort will be made to contact the village leader. If the village leader is available, he or she will be requested to facilitate contact and communication with identified households. A clinical nurse will review the consent form, answer questions, address parental concerns, and obtain informed consent from the parents of susceptible contact children (ages 6 mo-15 yrs) residing within a pre-determined meter radius of the index household. Following the acquisition of parental consent, blood samples will be collected from 10-25 contacts. Those parents (and children) who are unavailable to be consented (and bled) will be visited that same evening or the following morning. The clinical team will return to these homes approximately 5, 10 and 15 days after the initial visit to perform clinical assessments. The children bled on day 0 (initial specimen) will be re-bled on approximately day 15 (follow-up specimen). DV RT-PCR will be performed on all acute specimens. DV IgM/IgG ELISAs will be performed on paired initial and follow-up specimens.

An entomological team will collect mosquitoes, administer questionnaires, and perform insecticide spraying within the pre-determined meter radius of the index household (or an alternative specific pre-determined radius based on pre-study assessments of average household densities within the villages and experience gathered from Dengue Season 1). Another entomological team will collect mosquitoes but not perform insecticide spraying around the classroom and school bathroom areas of the index case.

e. Results:

KPSII Dengue Season1 and 2 report

NUMBER OF SUBJECTS ENROLLED/WITHDRAWN/APPROVED: Starting enrollment from 17 November 2003, 2,215 students (87% of a total of 2,538 target students) from 11 primary schools were enrolled in the study. Initially, meetings were conducted at those schools with the parents and guardians of those 2,538 target students invited (by an invitation letter) to attend to discuss and understand the research. A total of 1,667 parents and guardians

(approximately 65% of those invited) attended the meetings and a total of 1,520 students were enrolled immediately (91% of those attending the meetings). A total of 335 students were enrolled following the meetings at the public health offices, and a total of 213 students were enrolled following the house visits in the villages with the assistance and cooperation of the public health officials and village health workers. During the scheduled bleed in January 2004, a total of 36 students (1.6% of those enrolled) were found to have withdrawn. Of this number, 31 students (1.4% of those enrolled) withdrew due to movement of families out of the surveillance schools and 5 students (0.2% of those enrolled) due to the miscommunication nature in the families. The remaining number of the students is now 2,176.

SUMMARY OF RESULTS TO DATE: From the scheduled bleed in the total number of 2,176 students in January 2004, 36 students were found to have anemia. According to the pediatrician in Kamphang Phet Provincial Hospital, most of them were confirmed to have mild anemia with only 2 students confirmed to have moderate anemia.

During the school-based surveillance (Jan-March) 58 febrile students were drawn blood. Four of them were found RT-PCR positive (DEN1=1, DEN2=1 and DEN4=2). One of the four was ELISA positive. The ELISA was Secondary Dengue Infection. Of these four students, 2 were symptomatic and hospitalized. Another 2 were symptomatic but not hospitalized. Out of the 4 RT-PCR positive cases, we selected 2 index cases to cluster investigation. Another 2 RT-PCR positive cases were outside of our target study area. Also, 2 RT-PCR negative cases were selected for negative index cluster study. The cluster study showed that one of the two positive index clusters had 4 contact cases with asymptomatic dengue virus infection (DEN1=1, DEN3=1 and DEN4=2). Of these 4 asymptomatic cases, one was found to have primary dengue infection. The others were found ELISA negative. For the 2 negative index clusters we found that 3 contact cases had RT-PCR positive (DEN1=1, DEN2=1, and DEN3=1) on day 15.

School-based active surveillance for Year 2 conducted by village health workers visiting 11 primary schools during school days began in June 2004 (see Table1).

Table 1: Retention of Study Enrollees

School no.	1 June 04	1 Jul 04	1 Aug 04	1 Sep	1 Oct	1 Nov
01	130	132	131	131	130	130
02	267	266	266	263	262	262
03	369	358	358	358	358	358
04	134	133	132	130	129	129
05	106	102	100	98	97	97
06	168	168	167	166	166	166
07	192	191	190	192	192	192
08	183	181	180	180	180	179
09	129	129	129	129	127	127

10	228	227	227	227	227	227
11	189	187	186	186	186	186
total	**2095**	**2,074**	**2,066**	**2,060**	**2054**	**2053**

Since then until November 30; 1,747 children were evaluated due to school absence and 7,197 were the school absent periods. Of these, acute and convalescent sera were obtained from 505 students with acute or recent (reportedly within prior 7 days of evaluation) febrile illness. 44 children were positive for dengue RT-PCR on the acute specimens. Of these, six were hospitalized (see Table2), three with dengue fever caused by dengue virus serotype DEN-4 and one with DHF grade III caused by DEN-2, one with DHF (grade will be confirmed) by serotype 4 and one with DHF (grade will be confirmed) but RT-PCR negative. All six hospitalized dengue virus infection were acute secondary dengue infection.

Table 2: Summary of Symptomatic hospitalized cases

School No.	Grade of dengue infection (unconfirmed)	Serotype by RT-PCR	ELISA seroconversion	Hospital
02	DHF III	DEN-2	Acute 2° dengue infection	KPPPH
02	DF	DEN-4	Acute 2° dengue infection	KPPPH
02	DHF	DEN-4	Acute 2° dengue infection	KPPPH
03	DF	DEN-4	Acute 2° dengue infection	KPPPH
08	DF	DEN-4	Acute 2° dengue infection	KPPPH
11	DHF	NEG	Acute 2° dengue infection	KPPPH

In summary (see Table3), DEN-4 has been the most common serotype (38.63%) followed by DEN-2 (29.54%), DEN-3 (20.45%), DEN-1 (6.82%) and dual infection (4.54%). Of those 44 with positive RT-PCR, 25 were associated with dengue ELISA seroconversion. Schools 2 and 11 had a disproportionate number of dengue cases, though they were 25 kilometers apart. For ELISA seroconversion we have found 21 acute secondary dengue infection and 4 acute primary dengue infection (table 4). We have 3 cases of JE serology positive but all these 3 children had no neurological symptoms and signs and were returned to be normal within 7 days. They all had no JE vaccination short time before illness. We have 6 cases were positive ELISA seroconversion but RT-PCR negative. And we had 17 cases that RT-PCR were positive but the ELISA seroconversion negative. In these 17 cases we will confirm the HAI or sequence for the final results.

Table 3: Dengue Virus Serotypes Identified in the Schools by RT-PCR

School Number	Dengue RT-PCR Positive						Total
	DEN-1	DEN-2	DEN-3	DEN-4	DEN2 DEN4	DEN1 DEN3	
01		1	1	1			3
02		2		7	1		10
03		1	1	1			3
04	1		1			1	3
05		1					1
06		1	1				2
07	1						1
08	1	1	1	4			7
09				1			1
10		1		1			2
11		5	4	2			11
Total	3	13	9	17	1	1	44

Table 4. Dengue serotype and ELISA seroconversion results

DEN/JE IgM/IgG ELISA Interpretation	DV serotype by RT-PCR							Total
	DEN-1	DEN-2	DEN-3	DEN-4	Dual	Negative	Pending	
Acute primary DV infection		1	3			1	1	6
Acute secondary DV infection		6		14	1	1	3	25
Acute JE infection						3		3
Probable recent primary DV infection						1		1
No recent flavivirus infection	3	5	6	2	1	431	4	452
Pending results		1		1		16		18
Total	**3**	**13**	**9**	**17**	**2**	**453**	**8**	**505**

Results of Village-Based Cluster Investigations

In the village-based study from June to November we have had 14 positive clusters and 9 negative clusters totally 386 contacts (Table 5). We have 17 contact cases be positive for ELISA seroconversion (see Table 6). But we do not find ELISA positive in negative clusters. The RT-PCR in the positive clusters we have 7 RT-PCR positive in day 0 and 9 positive in day 15. For the negative cluster we have 3 RT-PCR positive in day 0 and 9 positive in day 15 (see Table 7). One contact in the positive cluster was admitted in the hospital because of DF.

Table 5 Number of contact cases in positive and negative clusters (Jun-Nov 04)

Cluster	Sex				Total	
	Female		Male			
	No.	% of Total	No.	% of Total	No.	% of Total
Negative	63	32.98	74	37.95	137	35.49
Positive	128	67.02	121	62.05	249	64.51
Total	191	100	195	100	386	100

Table 6 Serological results of the contact cases

ELISA interpretation	14 Positive Cluster contacts	9 Negative Cluster contacts
Acute Primary Dengue Infection	3	0
Acute Secondary Dengue Infection	13	0
Recent Secondary Dengue Infection	1	0
No recent flavivirus infection	230	136
Pending results*	2	1
Total	249	137

*Pending results
1. HAI assay pending
2. No Day 15 specimen due to relocation to another province

Table 7 RT-PCR of index cases and contact cases in 23 clusters (Jun-Nov 04)

Index cases	249 of positive contacts		137 of Negative contacts	
Day-1	Day 0	Day 15	Day 0	Day 15
14	7	9	3	9

f. Future plans:

Season 2 data analysis is underway. The study team is preparing for Season 3 activities.

4. Project Title: A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Children

a. Background:

The US Army seeks to acquire a licensed vaccine capable of protecting soldiers and their families from disease caused by infection with the dengue viruses. The Kingdom of Thailand shares this goal. For over 50 years the US Army has been active in developing and testing various vaccine candidates. This study represents the first use of the most promising Army dengue vaccine candidate in an overseas (Thailand), non-adult (children) population.

b. Objectives:

To demonstrate the WRAIR tetravalent dengue vaccine is safe and well-tolerated in a small number of Thai children between the ages of 5 and 9 years.

c. Methods:

1. Screen and enroll between 5 and 10 healthy, flavivirus naïve, Thai children between the ages of 5 and 9 years
2. Provide 2 doses of the WRAIR tetravalent dengue vaccine as outlined in the study protocol
3. Closely monitor the children following each dose of vaccine for safety and tolerability

d. Principal Investigators:

Stephen J. Thomas, MAJ, MC, USAMC-AFRIMS
Sriluck Simasathien, MD, Phramongkutklao Hospital (PMK), Bangkok, Thailand

Additional Study Personnel:

Rudiwilai Samakoses, MD (PMK Hospital)
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Kenneth H. Eckels, PhD (WRAIR)
J. Robert Putnak, PhD (WRAIR)
Celia Barberousse (GSK)

d. Results:

1. Seven children were ultimately enrolled and have received 1 dose of the WRAIR dengue vaccine

2. All children tolerated the vaccine well

3. Over 70 children who were screened but not enrolled received 2 doses of the Thai GPO JE vaccine as a benefit to participation

4. Tetravalent seroconversions were observed.

e. Future plans:

1. Seven children will receive a booster dose of the WRAIR dengue vaccine in a follow-up study planned for February 2005

2. Safety and tolerability will be monitored and measured

5. Project Title: A Phase I/II Trial of a Ttetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Infants

a. Background:

The US Army seeks to acquire a licensed vaccine capable of protecting soldiers and their families from disease caused by infection with the dengue viruses. The Kingdom of Thailand shares this goal. For over 50 years the US Army has been active in developing and testing various vaccine candidates. This study represents the first use of the most promising Army dengue vaccine candidate in an overseas (Thailand), infant population.

b. Objectives:

1. To demonstrate the WRAIR tetravalent dengue vaccine is safe and well-tolerated in a small number of Thai infants between the ages of 12 and 15 months.
2. To assess the immunogenicity of the dengue vaccine in terms of seroconversion 30 days post-dose 2 of dengue vaccine

c. Methods:

1. Screen and enroll 51 healthy, flavivirus naïve, Thai infants between the ages of 12 and 15 months
2. Provide 2 doses of the WRAIR tetravalent dengue vaccine as outlined in the study protocol
3. Closely monitor the infants following each dose of vaccine for safety and tolerability
4. Assess the immunogenicity of the dengue vaccine as outlined in the study protocol

d. Principal Investigators:

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Kenneth H. Eckels, PhD (WRAIR)
J. Robert Putnak, PhD (WRAIR)
Celia Barberousse (GSK)

e. Results:

Following the initial testing in U.S. adults and Thai children, the Walter Reed Army Institute of Research (WRAIR) live attenuated tetravalent dengue vaccine (Formulation 17) is currently being tested in this study in a randomized, observer-blind, controlled fashion to assess for the safety and immunogenicity of the vaccine in Thai infants. The infants received 2 doses of dengue/control vaccine six months apart followed by two doses of Japanese encephalitis (JE) vaccine. Fifty-one flavivirus-naïve (lack of prior exposure to dengue virus nor JE virus/vaccine) infants (12 – 15 months of age) have been randomized to receive either dengue vaccine or control vaccine (Varicella vaccine for dose 1 and Hemophilius B vaccine for dose 2). Fifty-one healthy male and female, flavivirus naïve infants were enrolled. Ninety-six (96) infants were screened for enrollment. Fifty-one (51) study subjects were selected and assigned to one of three cohorts (cohorts A, B and C). Dose 1 of dengue/control vaccination occurred on 29 Feb 04, 9 May 04 and 18 Jul 04 respectively. Cohorts A and B infants have received dose 2 of dengue/control vaccine on 9 May 04 and 14 Nov 04 respectively.

Screened	Cohort Enrollees	Dengue/ Control	Remarks
14	A (6 infants)	4 /2	1/10 of full dose (6 infants completed doses 1 and 2 of Dengue/Control vaccine)
28	B (15 infants)	10/5	Full dose (15 infants completed doses 1 and 2 of Dengue/Control vaccine)
54	C (30 infants)	20/10	Full dose (30 infants completed dose 1 of Dengue/Control vaccine) 1 infant was withdrawn from the study

f. Future plans:

The study is to conclude in March 2005 following which data will be cleaned in preparation for unblinding and release of data. Based on favorable safety and immunogenicity data, this vaccine will be further studied in Thailand in pediatric trials by AFRIMS in cooperation with the Thai MOPH and GSK.

6. Title of research project: Training and Workshops

a. Background:

The Department of Virology, Armed Forces Research Institute of the Medical Sciences (AFRIMS), Bangkok, Thailand, seeks to expand its diagnostic capabilities in South and

Southeast Asia by improving regional laboratory capabilities through the dissemination of diagnostic kits and the training of technical personnel.

b. Goals:

1. To create and improve the laboratory infrastructure of South and Southeast Asian regional laboratories specializing in infectious disease surveillance.
2. To provide the training of laboratory personnel (technicians and supervisors) working in South and Southeast Asia and beyond in infectious disease diagnostic techniques.

c. Activities:

The department conducted numerous on-site and in-house diagnostic training activities.

1. Over 50 student scientists from Srinakarinviroj University, Chulalongkorn University, Phramongkutklao Medical College, Faculty of Tropical Medicine and Mahidol University received 2 to 4 weeks of training at AFRIMS in diagnostic laboratory modalities.
2. On-site training (Kathmandu, Nepal) in the proper performance of the AFRIMS JE EIA and basic instruction in QA and QC principles was provided to representatives of numerous Nepali health institutions in an exercise facilitated by the the Environmental Health Project (EHP).
3. Training in the proper performance of the AFRIMS JE EIA and basic QA and QC principles was provided to visiting scientists from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, and National Institute of Pediatrics, Hanoi, Vietnam.
4. During the past year diagnostic kits and training were provided to the following laboratories:

B.P. Koirala Institute of Health Sciences, Dharan, Nepal
Nepal Public Health Laboratory, Kathmandu, Nepal
Teku Hospital, Kathmandu, Nepal
Bheri Zonal Hospital, Nepalgunj, Nepal
Institute of Medicine, Kathmandu, Nepal
ICDDRB, Dhaka, Bangladesh
Pasteur Institute, Ho Chi Minh City, Vietnam

7. Title of research project: Influenza Surveillance in Southeast Asia

a. Background:

Influenza is an important cause of morbidity and mortality among populations at the extremes of age. Continuous viral surveillance and isolation of influenza viruses provides important information for the creation of annual vaccine formulations based on the identification of new and emerging strains of influenza.

b. Goals:

1. To provide isolates of influenza virus collected in Southeast Asian countries as part of the global surveillance network for influenza, " Project Gargle".

2. To evaluate rapid diagnostic techniques at select sentinel sites in an attempt to validate these tests, increase sample submissions, and improve patient care.

3. To expand the network of participating institutions to include the U.S. Embassies of Southeast Asia.

c. Methods:

Samples were collected from patients with clinically suspected influenza infection (case definition includes fever or history of fever $\geq$38°C and two or more of the following symptoms: cough, sore throat, coryza, muscle aches, malaise/fatigue, or headache). Participating physicians and staff identified patients who met the case definition during routine clinic visits. Emphasis was placed on quality samples that may provide genetic data for future influenza vaccines rather than a large number of samples to be tested for incidence and prevalence data. Clinical history forms, including basic demographic and clinical information, were completed by the OPD nurse or AFRIMS research nurses. Throat swabs were collected and placed in viral media and stored at -70°C. All specimens were shipped on dry ice to AFRIMS, which in turn shipped the samples to Armstrong Laboratory, Brooks AFB. Rapid diagnostics for Influenza (FLU OIA) were field tested at the sites in Sankhlaburi, Thailand, the medical facilities of the US Embassy in Bangkok, Thailand, Kamphaeng Phet, Thailand and Kathmandu, Nepal. Laboratory test results were maintained and summarized by Project Gargle and CDC personnel.

d. Progress:

Sample collection for influenza surveillance was suspended during the Asian SARS epidemic to avoid placing health care personnel collecting and processing specimens at unknown risk. As a result, few samples were collected. A resurgence in SARS cases in Asia during the influenzae season will dicitate the level of influenzae surveillance activity.

F. Department of Retrovirology, AFRIMS FY04 Research Accomplishments

1. Title of research project: Screening and Evaluation of Potential Volunteers for a Preventive HIV-1 Vaccine Trial in Thailand (RV148, HSRRB Log No.).

a. Investigators:

Dr. Supachai Rerks-Ngarm, Dept. of Disease Control, MOPH;
COL Sorachai Nitayaphan, RTA Component, AFRIMS;
Prof. Dwip Kitayaporn, Mahidol University.

b. Objectives:

To evaluate adult Thai volunteers for eligibility and subsequent enrollment in a preventive HIV-1 vaccine trial (Phase III) of a prime-boost vaccine combination for the prevention of HIV infection.

c. Methods:

Volunteers who indicate an interest in participating in HIV vaccine research will receive information and education about the upcoming vaccine trial. They will be evaluated to see that they meet the eligibility criteria. They will receive counseling and education on HIV, aspects of participating in a HIV vaccine trial, and will be tested for HIV-1 infection by a standard ELISA & Western blot algorithm. Those volunteers who test positive for HIV will have CD4 enumeration and HIV viral load testing. Those that are eligible for the Phase III study and pass a test of understanding will be offered enrollment in a vaccine efficacy trial.

d. Results:

The protocol began enrolling volunteers as of 29 September 2003. Thus far over 12000 volunteers have been enrolled and screened.

e. Future Plans:

This protocol is projected to last approximately 2 years from time of enrollment in order to screen approximately 25,000 volunteers for the vaccine trial which has a sample size of 16,000.

2. Title of research project: A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp120 B/E (AIDSVAX® B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795).

a. Investigators:

Dr. Supachai Rerks-Ngarm, Dr. Supamit Chunsutthiwat- Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand
COL Sorachai Nitayaphan, RTA Component, AFRIMS
Prof. Dwip Kitayaporn, Mahidol Univesity
Assoc. Prof. Punnee Pitisuttithum, Mahidol University

b. **Objectives:**

Primary: To determine whether immunizations with an integrated combination of ALVAC-HIV (vCP1521) boosted by AIDSVAX® gp120 B/E prevent HIV infection in healthy Thai volunteers. Secondary: To determine whether immunization with this vaccine combination

results in reduced HIV viral load "set point" among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To determine whether immunization with this vaccine combination results in an increased CD4 count measured at viral load "set point" among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To confirm the safety of this vaccine combination in Thai volunteers.To evaluate whether participation in this HIV vaccine trial is associated with behavior change that may increase the risk of HIV infection.

c. **Methods:**

This will be a community-based, randomized, multicenter, double-blind, placebo-controlled clinical trial (vaccine:placebo = 1:1). Screening of potential volunteers will be carried out under a separate protocol entitled "Screening and evaluation of potential volunteers for a trial in Thailand of a candidate preventive HIV vaccine" (RV148). Eligible volunteers will be enrolled over approximately one year. The statistical assumptions of the study will require that 16,000 persons enroll into the study. Vaccinations for each individual will occur over a 24-week period (0, 4, 12, 24 weeks). Women will be tested for pregnancy and pregnant volunteers will not be vaccinated. The volunteers will be followed with HIV testing every 6 months for 3 years after immunization. Blood will be collected for plasma (for diagnostics and HIV-specific antibodies) at 0, 24 and 26 weeks, and every 6 months during the follow-up phase. The blood collection at 0 and 52 weeks will also be used for cryopreservation and archiving of PBMCs (for HIV-specific cellular immune responses). At week 24 and at each six-month follow-up visit, volunteers will have HIV testing, preceded by pretest counseling and followed (approximately 2-3 weeks later) by post-test counseling. Assessment of HIV risk behavior will be performed at baseline and at each 6-month follow-up visit. Education on risk behavior reduction will be given at each vaccination visit and at each post-test counseling visit.

d. **Results:**

Enrollment in this protocol began in October 2003, with the first volunteer injected on 20 October. As of December 2004, almost 8000 volunteers have been enrolled.

e. **Future Plans**:

This protocol will continue to enroll volunteers until the recruitment goal is met. Volunteers will be followed for 3 ½ years after vaccination.

III. APPENDICES:

A. PERSONNEL ASSIGNED UNDER AGREEMENT

Department of Administration

1. Ms.Bang-on Kesdee
2. Mr.Weerasak Yeephu
3. Mr.Sompol Boonnak
4. Ms.Pattrapan Jullasing
5. Mr.Puwanai Sangsri
6. Ms.Geerati Sornwattana
7. Mrs.Wichayada Wattanatom
8. Ms.Yingluk Apisitsaowapa
9. Ms.Tippawan Tephassadin na
10. Mr.Somporn Krasaesub
11. Mr.Theerasak Ponepan
12. Mr.Prinya Yoophasook
13. Mrs.Khanitha Rojanastien
14. Ms.Pasana Chuanak

Department of Logistics

15. Mr.Nipat Promchart
16. Mr.Sawadi Boonnak
17. Mr.Charan Kajeechitr
18. Mr.Thongchai Duangkeaw
19. Mr.Boonthum Jamjank
20. Mr.Komson Boonnak
21. Mr.Somporn Pinpo
22. Mr.Chatchai Sang-ngen
23. Mr.Prasitchai Kruysawat
24. Mr.Yuthana Seemat
25. Mr.Sawet Amnuay
26. Mr.Patraphum Kwanyou
27. Mrs.Anchisa Maneenun
28. Ms.Nongnoot Chaowaratana
29. Ms.Panisara Puangsilpa
30. Mr.Tharanut Thanatepaisansakun
31. Ms.Yuwadee Sae-Yong
32. Ms. Saruta Anukul

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34. Ms.Amporn Chalouydumrong
35. Ms.Nillawan Buathong
36. Mr.Chaiyawat Mathavarat
37. Mr.Prasit Sookto
38. Ms.Nitima Chanarat
39. Ms.Werawan Chonarom
40. Mrs.Somchit Tulyayon
41. Ms.Srisombat Wannaying
42. Ms.Apassorn Saelim
43. Ms.Sbaitip Sriwichai
44. Ms.Duankamon Siludjai
45. Ms.Monthicha Kongthaisong
46. Ms.Ratsuda Yapom
47. Ms.Suthatta Hanwisai
48. Mr.Sittidech Surasri
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57. Ms.Panor Srisongkram
58. Mr.Surind Sisiranond
59. Mr.Somsak Imlarp
60. Mr.Pairote Tararut
61. Mr.Wichien Sa-Nguansuk
62. Mr.Prachakkra Panthusiri
63. Ms.Wallika Kulthongkum
64. Mr.Wanchai Inpho
65. Mrs.Pannarat Chuakanubon
66. Mrs.Rungkarn Hangsuwan
67. Dr.Chusak Pimgate
68. Mr.Yongyuth Poonpanichupatam
69. Mr.Thanawat Boonnak
70. Ms.Rattiya Wannawongy

71. Ms.Prinyada Rodpradit
72. Ms.Piyawan Chinnawiropisan
73. Mrs.Wilaiwan Sridadeth
74. Mr.Wiangchai Watcharanirun
75. Mr.Wicha Panyalikhit
76. Mr.Winai Kaneechit
77. Mr.Chaleaw Sangchan
78. Ms.Thidarat Intararit
79. Ms.Nualanong Akaraputtiporn

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81. Mr.Thonglor Detkokao
82. Mr.Sawaeng Sripakdee
83. Mr.Phatcharaphon Jaikla
84. Mr.Samruay Jecksaeng
85. Mr.Dejmongkol Onchompoo
86. Mr.Manop Pooyindee
87. Ms.Choosri Sangsri
88. Mr.Surayuth Seegaewin
89. Mr.Somkid Tosawong
90. Mr.Manas Kaewsurind
91. Mr. Yongyuth Kongkaew
92. Mr.Vittavat Sankalee
93. Mr.Mana Saithasao
94. Ms.Anchalee Pothipoch
95. Mr.Bamrung Chaikwang
96. Ms.Siriwan Korpaiboonkij
97. Mr.Alongkorn Hanrujirakomjorn
98. Mr.Sornchai Jansuwan
99. Mr.Amnaj Andang
100. Mr.Suvit Boonkali
101. Mr.Sarayuth Chienrum
102. Mr.Sakda Sanon
103. Ms.Kwanpracha Insansueb
104. Mr.Chaisit Pornkhuviwat
105. Mr.Wuthichai Puenchompu
106. Ms.Rawiwan Imebsin
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132. Ms.Nittaya Khlaimanee
133. Mr.Narong Ponsa
134. Ms.Nongnuch Maneechai
135. Mr.Udom Kijchalao
136. Ms.Suda Ratanawong
137. Mrs.Prachong Panthusiri
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139. Mrs.Namtip Trongnipatt
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142. Ms.Wilasinee Akkagraisee
143. Ms.Kwanta Chayapumb

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149. Ms.Chittima Pitarangsi
150. Ms.Ajchara Aksomboon
151. Mr.Boonchai Wongsatitiwilairoong
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153. Mrs.Wonlana Teerapolumpun
154. Mr.Supin Pankote
155. Mr.Suchart Thepsanan
156. Mr.Prasert Meesuksabai
157. Ms.Prapatcha Chitsoonthornrat
158. Mrs.Tanintorn Adeedto
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160. Ms.Rapida Padmasankha
161. Mr.Pakornpat Supanich
162. Mr.Nan Chen
163. Mrs.Tippa Wongstitwiliroong
164. Ma.Nopparat Saeier
165. Ms.Nataya Ruamsap
166. Ms.Umaporn Suksawad
167. Ms.Piyarat Pootong
168. Ms.Kaewkanya Nakjarung
169. Ms.Piyanate Sunyakumthorn
170. Mr.Prani Ratarasarn
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182. Ms.Vilaiwan Tungsakul
183. Ms.Kanya Pumratana
184. Mrs.Sujitra Santantivongchai
185. Mr.Thiti Inngam
186. LTG.Pricha Singharaj
187. Mrs.Suchada Chinaworapong
188. Mrs.Puangmalee Buapunth
189. Mr.Attapon Triampon
190. Ms.Pirinat Chalempak
191. Ms.Sopana Chatnibandhu
192. Ms.Suchada Sukhumvitaya
193. Ms.Sakuna Suksawad
194. Ms.Nongluck Sangnoe
195. Ms.Thunyasuta Prasit
196. Ms.Nampueng Sirijongdee
197. Ms.Matta Thongtaluang
198. Mr.Siriwat Akapirat
199. Mr.Sunboon Kaewprapun
200. Ms.Ajchariyarat Sangdara
201. Mrs.Sumonsak Thojun
202. Mr.Surat Jongrakthaitae
203. Ms.Threeraporn Thamjamras
204. Mr.Sutee Srisukwattana
205. Mr.Nitit Isravudhakul
206. Ms.Surapee Suntavaruk

****

B. PUBLICATIONS 2004

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C. ABSTRACTS 2004

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